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insight into their possible connections

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Mitochondrial dysfunction and altered dNTP levels: Insight into their possible connections

Ph.D. Thesis

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January 2009

Content

Preface

This Ph.D. thesis is based on work carried out at the Department of Science, Systems and Models at Roskilde University from 2005 to 2009 under the supervision of Professor Lene Juel Rasmussen.

The Ph.D. project has aimed to provide information about the involvement of DNA precursors, deoxyribonucleotide triphosphates (dNTP), in maintaining genomic stability. The primary focus of the study has been on the regulation of dNTP in cells with dysfunctional mitochondria. Furthermore, the role of dNTP levels following DNA damage has been studied in *Saccharomyces cerevisiae*. In addition I have worked on an *In Silico* sorting strategy to predict mitochondrial proteins amongst hypothetical proteins and finally I have demonstrated that the polycyclic aromatic hydrocarbon anthanthrene is mutagenic despite general belief.

The results are presented in three published papers (Paper I, II, and V) and two unpublished papers (Paper III (submitted) and IV (in preparation)). A mini-review has been prepared describing the possible links between mitochondrial dysfunction and dNTP regulation

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January 2009

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Summary

An available and balanced supply of the precursors of DNA, deoxyribonucleotide triphosphates (dNTP), is essential for genomic stability. Mitochondria are unique organelles in that they contain a mitochondrial genome and a dNTP pool that is separated from the rest of the cell. In mammals, mitochondria furthermore contain specific constituents of the dNTP salvage pathway, which allows independent synthesis of dNTP.

In the present thesis, an intricate link between mitochondria and the regulation of the cellular dNTP pools has been demonstrated. Thymidine kinase 2 (TK2) is a rate-limiting enzyme of the mitochondrial salvage pathway. Despite decreased activity of the kinase, human colon cancer cells were able to maintain a balanced mitochondrial dNTP pool. This strongly suggests that mitochondria of dividing cells are not dependent on mitochondrial synthesis of dNTP. Conversely mitochondrial dysfunction caused by depletion of the mitochondrial genome has been shown to affect the cytosolic dNTP pool, causing both an imbalance and decrease in concentrations. Concurrent with the dNTP imbalance, chromosomal instability was also demonstrated, emphasizing that imbalanced dNTP pools are mutagenic.

In *Saccharomyces cerevisiae* an increase in dNTP pools following DNA damage increases mutation frequencies, but also cell survival. Increased survival after DNA damage is mediated by Translesion DNA Synthesis (TLS) and the altered levels of dNTP is a prerequisite for the DNA damage by-pass.

Recent work has demonstrated that the mitochondrial proteome is not complete and it is very likely that novel mitochondrial proteins are yet to be characterized. We have constructed an *In Silico* sorting strategy that can be used on hypothetical proteins in order to find the candidates most likely to have a role in the investigated context. Even though we examined human hypothetical proteins

for existance of uncharacterized mitochondrial proteins, but the sorting strategy may also be used for other organelles in other organisms.

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants, formed during incomplete burning of coal, oil and gas. Previously it was believed that only PAHs with either bay or fjord motifs were mutagenic. We have demonstrated that the PAH anthanthrene induces DNA damages despite it lack of these motifs. DNA damages induced by anthanthrene metabolites where demonstrated to be primarily repaired by Nucleotide Excision Repair.

Summary in Danish

En tilgængelig og balanceret forsyning af deoxyribonucleotide triphosphater (dNTP) er afgørende for genomiske stabilitet. Mitokondrier er unikke organeller idet de indeholder et mitokondrielt genom og en dNTP pool som er adskilt fra resten af cellen. I pattedyr indeholder mitokondrier desuden specifikke bestanddele af salvage pathwayen, som gør det muligt for mitokondrier at producere dNTP uafhængigt af resten af cellen.

I denne afhandling påvises en sammenhæng mellem mitokondrier og regulering af cellulære dNTP pools. Thymidin kinase 2 (TK2) er et rate-limiting enzym der fungerer i den mitokondrielle salvage pathway. Trods nedsat TK2 aktivitet, var menneskelige kolon cancerceller i stand til at opretholde en balanceret mitokondriel dNTP pool. Dette tyder på at mitokondrier i delende celler ikke er afhængige af den mitokondrielle syntese af dNTP. En mitokondriel dysfunktion, forårsaget af en mangel af det mitokondrielle genom, påvirker omvendt den cytosoliske dNTP pool og skaber både en ubalance og et fald i koncentrationen af dNTP. Samtidig med en dNTP ubalance, blev forekomst af kromosomal ustabilitet demonstreret, hvilket understreger at ubalancerede dNTP pools er mutagene.

I *Saccharomyces cerevisiae,* medfører forekomsten af DNA skader en forøgelse af koncentrationen af dNTP. Dette resulterer i en øget mutationsfrekvens, men er også essentiel for cellens overlevelse. Øget overlevelse efter DNA-skader er medieret af Translesion DNA Synthesis (TLS) og det ændrede niveau af dNTP er en forudsætning for by-pass af DNA skader

Det mitokondrielle proteome er ikke komplet og det er meget syndsynligt, at nye mitokondrielle proteiner mangler at blive karakteriseret. Vi har konstrueret en *in silico* sorterings strategi, der kan bruges på hypotetiske proteiner med henblik på at finde de kandidater, som mest sandsynligt er udtrykt i den undersøgte

kontekst. Selv om vi har undersøgt tilstedeværelsen af ukarakteriserede mitokondrielle proteiner i mennesker, kan den beskrevne sorterings strategi også anvendes i andre organismer og for andre organeller.

Polycykliske aromatiske kulbrinter (PAH) er miljøforurenende stoffer, der dannes ved ufuldstændig forbrænding af kul, olie og gas. Tidligere mente man det, at kun PAH'er med enten Bay eller Fjord strukturmotiver var mutagene. Vi har påvist at PAHen anthanthren inducerer DNA-skader på trods af en mangel på disse motiver. Ydermere har vi kunnet demonstrere at DNA-skader forårsaget af anthanthren primært repareres af Nucleotide Excision Repair (NER).

List of papers

This thesis is based on the following papers:

Paper I: Desler, C., Munch-Petersen, B. and Rasmussen, L.J. (2006) The role of mitochondrial dNTP levels in cells with reduced TK2 activity. *Nucleosides, nucleotides & nucleic acids*, **25**, 1171-1175.

Paper II: Desler, C., Munch-Petersen, B., Stevnsner, T., Matsui, S., Kulawiec, M., Singh, K.K. and Rasmussen, L.J. (2007) Mitochondria as determinant of nucleotide pools and chromosomal stability. *Mutation research*, **625**, 112-124.

Paper III: Desler, C., and Rasmussen, L.J.Increased concentrations of dNTP following DNA damage determines the TLS response in *Saccharomyces cerevisiae* Manuscript in preparation

Paper IV: Desler, C., Suravajhala, P., Sanderhoff, M., Rasmussen, M. and Rasmussen, L.J. (2009) *In Silico* screening for functional candidates amongst hypothetical proteins. *BMC Bioinformatics,* Revision Submitted.

Paper V: Desler, C., Johannessen, C. and Rasmussen, L.J. (2008) Repair of DNA damage induced by anthanthrene, a polycyclic aromatic hydrocarbon (PAH) without bay or fjord regions. *Chemico-biological interactions*. **177**, 212-217.

Abbreviations

Ala	Alanine
AP	Apurinic
Ara-T	Thymidine-β-D-arabinoside
Arg	Arginine
Asn	Asparagine
ATP	Adenosine triphosphate
BER	Base Excision Repair
CIN	Chromosomal instability
Coq3	Coenzyme Q3 homologue
cSHMT	Cytosolic SHMT
CTP	Cytidine triphosphate
dADP	Deoxyadenosine 5'-diphosphate
dAMP	Deoxyadenosine 5'-monophosphate
dATP	Deoxyadenosine 5'-triphosphate
dCDP	Deoxycytidine 5'-diphosphate
dCMP	Deoxycytidine 5'monophosphate
dCTP	Deoxycytidine 5'-triphosphate
dCyd	Deoxycytidine
dGDP	Deoxyguanosine 5'-diphosphate
dGK	Deoxyguanosine kinase
dGMP	Deoxyguanosine 5'-monophosphate
dGTP	Deoxyguanosine 5'-triphosphate
DHODHase	Dihydroorotae dehydrogenase
DNA	Deoxyribonucleic acid
dNDP	Deoxyribonucleoside diphosphate
dNMP	Deoxyribonucleoside monophosphate
dNTP	Deoxynucleotide 5'- triphosphate
dTDP	Deoxythymidine 5'-diphosphate
dThd	Deoxythymidine
dTMP	Deoxythymidine 5'-monophosphate
dTTP	Deoxythymidine 5'-triphosphate
dUDP	Deoxyuridine diphosphate
DUF	Domain of unknown function
ETC	Electron transport chain
FACS	Fluorescence-activated cell sorter
Fe/S protein	Iron-sulfur protein
FMP32	Found in mitochondrial proteome protein 32
GGR	Global Genomic Repair
His	Histidine
hMLH1	Human MutL Homologue 1
hMSH2	Human MutS homologue 2
HNPCC	Hereditary nonpolyposis colorectal cancer
HPLC	High performance liquid chromatography
HSP70	Heat Shock Protein 70
HSP90	Heat Shock Protein 90
HUGO	Human Genome Organization
LOH	Loss of heterozygosity
MDS	mtDNA depletion syndrome
MIN pathway	Microsatellite instability pathway
MMR	Mismatch Repair
MMS	Methyl-methanesulfonate
MNGIE	Mitochondrial neurogastrointestinal encephalomyopathy
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
mRNA	Messenger Ribonucleic acid
mSHMT	Mitochondrial SHMT
MSI	Microsatellite instability
mtDNA	Mitochondrial DNA
mtNOS	Mitochondrial nitric oxide synthase
NADH	Nicotinamide-adenine dinucleotide
nDNA	Nuclear DNA
NER	Nucleotide Excision Repair
NO	Nitric oxide
PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction

PT pore	Permeability transition pore
RER	Recombinational repair
RNR	Ribonucleotide reductase
ROS	Reactive oxygen species
SAM	S-adenosyl-L-methionine
Ser	Serine
SHMT	Serine hydromethyltransferase
SKY	Spectral Karyotyping analysis
SMART	Simple Modular Architecture Research Tool
TCR	Transcription Coupled Repair
THF	Tetrahydrofolate
TK1	Thymidine kinase 1
TK2	Thymidine kinase 2
TLS	Translesion synthesis
TOM complex	Transporter Outer Membrane complex
Trm112p	tRNA methyltransferase 112p
TRP	Tetratricopeptide repeats
UMP	Uridine monophosphate
UTP	Uridine triphosphate
XP	Xeroderma pigmentosum
XPA	Xeroderma pigmantosum complementation group A
XPC	Xeroderma pigmantosum complementation group C

Mini review

Mitochondrial dysfunction and altered dNTP levels: Insight into their possible connections

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Abstract

Mitochondrial dysfunction has been demonstrated to result in decreased levels as well as imbalance of cellular deoxyribonucleotide triphosphate (dNTP) pools in dividing human cells. While such an affect on the dNTP pool are known to cause genetic instability in the nuclear genome, the nature of the link between mitochondria and regulation of dNTP pools is unknown. In this mini review, we describe possible pathways, whereby a mitochondrial dysfunction can influence regulation of the cytosolic dNTP levels. We argue that mitochondrial dysfunction does not induce imbalances of cytosolic dNTP by deficient export or excessive import of deoxyribonucleotides to the organelle. It is more likely that dysfunctional mitochondria mediate inhibition of the *de novo* synthesis of precursors of dNTP through inhibited activity of the mitochondrial enzyme dihydroorotate dehydrogease or by a decreased production of mitochondrial one-carbon units, both important for the *de novo* synthesis of dNTP. Furthermore, an erratic regulation of NO produced in mitochondria, is suggested to affect both intra mitochondrial and cytosolic regulatory pathways important for *de novo* dNTP synthesis.

The biological role of a link between mitochondria and regulation of the dNTP levels is unknown, but could participate in the progress of apoptosis. In the case of a mitochondrial dysfunction, imbalanced dNTP pools may define a role for mitochondria in the progression of cancer

Introduction

Mitochondria are semiautonomous organelles present in almost all eukaryotic cells ranging from a single copy to several thousands. Important mitochondrial functions include ATP production by oxidative phosphorylation, β-oxidation of fatty acids and metabolism of amino acids and of lipids. Furthermore, mitochondria have a prominent role in apoptosis. Most human cells contain between 10^3 and 10^4 copies of circular mitochondrial DNA (mtDNA) molecules [Cummins 1998].

The organelle is double-membrane enclosed and an electron transport chain (ETC) maintains an electrochemical potential gradient, between the inter-membrane space and the matrix of the mitochondria. The ETC is constituted of four membrane bound enzyme complexes (complex I-IV), two electron carriers (ubiquinon and cytochrome c), and is located in the inner membrane of the mitochondria. The most often mentioned utilizer of the electrochemical gradient is the ATP synthase (complex V) generating ATP by oxidative phosphorylation. Mitochondrial dysfunction that results in depolarization of the inner mitochondrial membrane causes changes of cellular functions that can manifest as a variety of pathologies. When the electrochemical gradient is compromised, the cell suffers from lower production of ATP and some well characterized pathologies resulting from a mitochondrial dysfunction are directly linked to an insufficient ATP production [DiMauro and Schon 2003]. Mitochondria contain genome(s), separate from the nuclear genome that encodes 37 polypeptides, 13 of those encoding subunits, critical for ETC function [Wallace 1999; DiMauro and Schon 2003]. Mitochondrial dysfunction that results in depolarization of the

mitochondrial membrane can, therefore, be the result of mutations in either the mitochondrial or nuclear genome and be acquired or follow both Mendelian or maternally inheritance. In the following we will focus on mitochondrial dysfunction resulting in depolarization of the mitochondrial membrane, and any reference to mitochondrial dysfunction will, unless otherwise stated, mean mitochondrial dysfunction resulting in depolarization of the mitochondrial membrane.

The cellular content of deoxyribonucleotide tri-phosphate (dNTP) is sequestered into two pools, a mitochondrial and a cytosolic, separated by the mitochondrial double membrane [Bogenhagen and Clayton 1977]. Both a *de novo* and a salvage pathway carry out synthesis of cytosolic dNTP, while only a salvage pathway is available for mitochondria. The synthesis of mitochondrial dNTP occurs throughout the cell cycle whereas synthesis of cytosolic dNTP is restricted to synthesis in proliferating cells and little or no synthesis in resting or non-proliferating cells. A balanced supply of dNTP is required for correct DNA repair and replication [Meuth 1989]. Imbalance of the dNTP pools can induce base substitutions, frameshift mutations, delay of replication fork progression and DNA replication and enhance the frequency of fragile sites [Mathews *et al.,* 2006; Ke *et al.,* 2005; Reichard 1988; Bebenek and Kunkel 1990; Jacky *et al.,* 1983; Kunz and Kohalmi 1991; Wickremasinghe and Hoffbrand 1980; Golos and Malec 1991]. We have recently demonstrated how depletion of the mitochondrial genome, in two different human cancer cell lines, results in decreased levels as well as imbalance of cellular dNTP pools when the cells are in exponential phase. Imbalanced cytosolic dNTP pools causes genetic instability in the nuclear

genome and in accordance, we show that mitochondrial dysfunction results in chromosomal instability [Desler *et al.,* 2007]. We did not find any straightforward correlation between ATP levels and dNTP pools indicating that mitochondrial dysfunction can affect the regulation of dNTP levels and that this relationship, is not necessarily controlled by variations in ATP levels.

In this review we describe possible pathways, whereby a mitochondrial dysfunction can influence regulation of the cytosolic dNTP levels. These include a reduced production of precursors for *de novo* synthesis of dNTP mediated by inhibition the activity of the mitochondrial localized enzyme dihydroorotate dehydrogenase or an insufficient production of one carbon units from the mitochondria. Furthermore, we will describe whether mitochondrial dysfunction can result in an erratic regulation of NO produced in mitochondria, affecting both intra mitochondrial and cytosolic regulatory pathways important for *de novo* dNTP synthesis.

We will briefly suggest a role for the link between mitochondria and dNTP regulation in a non-pathological context and discuss the biological role of the link between dysfunctional mitochondria and a dNTP imbalance in the propagation of cancer.

Flux of deoxyribonucleotides between mitochondria and the cytosol is unlikely to cause a cytosolic dNTP imbalance

The replication of mtDNA is not synchronized with the synthesis of nuclear DNA (nDNA) [Davis and Clayton 1996] and the organelle therefore requires a

constant supply of dNTP. Mitochondria are able to synthesize dNTP by a specific salvage pathway where imported deoxyribonucleosides are phosphorylated by the two rate-determining deoxyribonucleoside kinases: thymidine kinase 2 (TK2) and deoxyguanosine kinase (dGK) yielding deoxyribonucleoside mono-phosphates (dNMP), which in turn can be further phosphorylated to their corresponding di- and tri phosphates (dNDP and dNTP) or be dephosphorylated to deoxyribonucleosides again by mitochondrial deoxyribonucleotidases [Eriksson *et al.,* 2002; Bianchi and Spychala 2003]. The mitochondrial inner membrane is impermeable to charged molecules and consequently, there is no direct exchange of deoxyribonucleotides between the cytosolic and mitochondrial compartments. However, several observations have indicated a facilitated transport between the two compartments [reviewed in Mathews and Song 2007]. It can, therefore, be speculated that the flux of deoxyribonucleotides between cytosol and dysfunctional mitochondria can result in a cytosolic dNTP imbalance.

Export of deoxyribonucleotides from the mitochondrial to the cytosolic compartment has been demonstrated. In cultured cells lacking essential enzymes of the cytosolic salvage pathway, an exogenous source of labeled thymidine was imported into the mitochondria and phosporylated exclusively by the mitochondrial salvage pathway, resulting in a mitochondrial pool of labeled dTTP. An efflux of labeled dTTP to the cytosolic compartment and an incorporation of labeled dTTP into nDNA could be demonstrated, confirming an export of mitochondrial deoxyribonucleotides. Even though the cells lacked a functional cytosolic salvage pathway the contribution of the labeled dTTP only

constituted a fraction of the cytosolic pool of dNTP, indicating a strong dominance of dNTP produced by the *de novo* pathway, demonstrating that the mitochondrial contribution to the cytosolic pool of dNTP is negligible [Pontarin *et al.,* 2003]. Conversely, import of the precursor of dTTP, deoxythymidine mono-phosphate (dTMP), has been demonstrated from the cytosolic- to the mitochondrial compartment using pulse-chase experiments with radioactively labeled dTMP. Within the organelle, some of the dTMP was phosphorylated to dTDP and dTTP. The mitochondrial import of cytosolic dTMP, was unaffected by inhibition of mitochondrial ATP production or inhibition of the ETC, which are hallmarks of depolarization of the mitochondrial membrane [Ferraro *et al.,* 2006].

In non-dividing tissue, loss of function mutations of TK2 has been demonstrated to result in mitochondrial dNTP imbalance and consequently deletions and depletions of mtDNA [Saada *et al.,*2001]. Furthermore, even subtle impairment of TK2 activity has been reported to result in decreased mitochondrial dNTP pool [Wang *et al.,* 2003]. Using a human cervical cancer cell line that can be induced to express lower levels of TK2, it was possible to reduce the activity of TK2 in the cell with 47% [Desler *et al.,* 2006]. The activity was assayed by the ability of TK2 to phosphorylate thymidine-β-D-arabinoside. In dividing cells, a reduced activity of TK2 induced a minor increase of the mitochondrial dCTP pool, while the dTTP, dATP and dGTP pools where unaffected. This indicates a mitochondrial import of cytosolic deoxyribonucleotides. However, in cells with reduced TK2 activity, no difference was measured in the cytosolic dNTP pools and it was concluded the cytosolic synthesis of dNTP in dividing cells can

sufficiently supply both nuclear and mitochondrial replication without inducing cytosolic dNTP imbalance [Desler *et al.,* 2006].

Deoxyribonucleotides synthesized by the mitochondrial salvage pathway are exported to the cytosol, however, the mitochondrial contribution to the cytosolic dNTP pool has been demonstrated to be negligible [Pontarin *et al.,* 2003]. Furthermore, the import of dTMP through the mitochondrial membrane has been demonstrated not to be affected by a depolarization of the mitochondrial membrane induced by various respiratory inhibitors [Ferraro *et al.,* 2006]. Finally, reduced activity of TK2, and thereby increased cellular dependence on cytosolic dNTP, does not affect the cytosolic pool of dNTP in dividing cells [Desler *et al.,* 2006]. Together this strongly indicates that in dividing cells, a mitochondrial dysfunction is unlikely to cause a cytosolic dNTP imbalance by deficient export or excessive import of deoxyribonucleotides.

Dihydroorotate dehydrogenase links mitochondria to the *de novo* pyrimidne biosynthesis

The flavoenzyme dihydroorotae dehydrogenase (DHODHase) catalyzes the conversion of dihydroorotate to orotate by oxidation, making DHODHase an integral step of the *de novo* synthesis of pyrimidines. Subsequent catalytic steps convert orotate into uridine monophosphates which can be further converted to UTP and CTP, and ultimately, dTTP and dCTP. DHODHase is located in the inner membrane of the mitochondria with the active site facing the inner membrane [Jones 1980; Rawls *et al.,* 2000]. DHODHase is functionally connected to the

electron transport chain by a flavin prosthetic group that couples dihydroorotate oxidation to respiratory ubiquinone reduction [Bader *et al.,* 1998]. From ubiquinol, the reduced form of ubiquinone, the flow of electrons continues through the electron transport chain.

As a result of the connection between DHODHase and the electron transport chain, it has been suggested that any dysfunction of the electron transport chain – lack of oxygen, presence of inhibitors or inherited and acquired defects of complex III and IV, would automatically entail impairments of the *de novo* UMP synthesis and a subsequent decrease in the *de novo* synthesis of pyrimidines and, thereby, the cytosolic nucleotide pools [Löffler *et al*., 1997]. Along this line, it has been suggested that mitochondrial dysfunction can lead to imbalance of the cytosolic dNTP pool and this is caused by inhibition of the DHODase [Singh 2004]. The relationship between DHODHase and the ETC has been described using the two ETC inhibitors, rotenone and sodium cyanide on mitochondria isolated from rat liver, kidney and heart. Rotenone inhibits the transfer of electrons from complex I of the ETC to ubiquinone, whereas sodium cyanide inhibits complex IV. In isolated mitochondria, treatment with sodium cyanide abolishes the activity of DHODHase while treatment with rotenone does not seem to have an effect [Löffler *et al.,* 1997]. This indicates that only an inhibition or impairment of the complexes of ETC that causes a build up of ubiquinol, has an inhibitory effect on the DHODase. A functional ETC is not only necessary for correct function of DHODHase, but also for correct localization of the protein. DHODHase is encoded by nDNA and the import into the membrane of the mitochondria is dependent on a targeting signal in the N-terminal segment of the

protein. It has been demonstrated that import of rat DHODHase into yeast mitochondria was abolished by an uncoupling of the mitochondrial membrane indicating the requirement of a membrane potential for correct positioning of DHODHase [Rawls *et al.,* 2000].

The importance of a functional DHODHase for the *de novo* synthesis of pyrimidines is emphasized by the effect of inhibitors of DHODHase. Leflunomide and brequinar are two examples of DHODHase inhibitors that bind to the quinone-binding site of DHODHase, thereby, blocking interaction between ubiquinone and the flavin prostethic group of DHODHase [Liu *et al.,* 2000]. Treatment of human lymphocytes with leflunomide or brequinar arrest the cells in G1 phase and inhibit both RNA and DNA synthesis [Chong *et al.,* 1996; Rückermann *et al.,* 1998; Greene *et al.,* 1995]. The inhibitory effects are suppressed by addition of uridine, which can be salvaged to UMP whereby the *de novo* synthesis of pyrimidines is by-passed. Treating the human leukemic cell line CCRF.CEM with leflunomide or brequninar cause a significant reduction in the levels of CTP and UTP, while the levels of purine nucleotides are unaffected in the case of lefunomide or increased in the case of brequinar [Cherwinski *et al.,* 1995].

Chloramphenicol is an antimicrobial agent that inhibits mitochondrial protein synthesis [Storrie and Attardi 1973]. Treatment of cells with chloramphenicol therefore mimics mitochondrial dysfunction and impaired ETC activity. Treatment of chick embryo cells with chloramphenicol inhibits activity of DHODHase as well as cell growth [Grégoire *et al.,* 1984]. Growth of Ehrlich Ascites tumor cells under hypoxic conditions inhibited the ETC and caused

reduced activity of the DHODase and G1 arrest [Löffler 1980]. For both the chloramphenicol treated chick embryo cells and hypoxic Ehrlich Ascites tumor cells, growth inhibition was reversed by addition of pyrimidines to the growth media. This indicates that mitochondrial dysfunction affecting the ETC has an inhibitory effect on DHODHase comparable to inhibition with leflunomide or brequinar. This is substantiated by the fact that cultured cells devoid of mtDNA are auxotrophic for pyrimidines and must be routinely grown in the presence of a uridine supplement [King and Attardi 1989].

Mitochondrial dysfunction affecting the ETC is likely to cause decrease of pyrimidine nucleotides mediated by an inhibition of DHODHase activity. As a result of the decreased levels of cytosolic pyrimidine nuclotides, an inhibition of both RNA and DNA synthesis is likely to entail. Imbalanced dNTP pool resulting from inhibition of DHODHase has been proposed [Singh 2004]. However, no experimental evidence has been provided to support this theory, but since *de novo* synthesis of cytosolic dCTP and dTTP requires a functional DHODHase, the relationship is possible.

Mitochondrial production of one-carbon units links mitochondria to the *de novo* purine biosynthesis

Serine is a major source of one-carbon units required for the synthesis of glycine, thymidylate, methionine, several methylation reactions and, most important for this review, purine synthesis. In mammals, serine is derived from the diet and is synthesized from glycolysis via 3-phosphoglycerate. Serine is reversible

converted into glycine in a process catalyzed by serine hydromethyltransferase (SHMT) where a methyl group is transferred from serine to tetrahydrofolate (THF), yielding glycine and 5,10-methyleneTHF [Schirch 1982]. There are two isoenzymes of SHMT, a cytosolic (cSHMT) and a mitochondrial (mSHMT) [Garrow *et al.,* 1993]. It is generally believed that the process catalyzed by mSHMT is the primary pathway for conversion of serine to glycine and 5,10-MethyleneTHF. This is substantiated by studies using Chinese Hamster Ovary (CHO) cells lacking mSHMT activity where a 15-fold accumulation of intracellular serine concentration over wildtype CHO cells has been demonstrated. Despite the increased concentration of serine, the net flux through cSHMT was demonstrated to be in the direction of serine synthesis [Narkewicz *et al.,* 1996]. Furthermore, CHO cells deficient in mSHMT activity are glycine auxotrophs [Pfendner and Pizer 1980], and the auxotrophy can be suppressed by transfection with a human version of mSHMT, suggesting that the primary role of mSHMT but not cSHMT is to generate glycine [Stover *et al.,*1997].

Within the mitochondrial compartment, 5,10-methyleneTHF is converted into formate in a series of enzymatic conversions initiated by the conversion of 5,10-methyleneTHF into 5,10-methenyltetrahydrofolate in a NAD+ dependent reaction catalyzed by methylenetetrahydrofolate dehydrogenase (methylene-THF dehydrogenase). Formate can be exported from mitochondria to cytosol, where it is converted to 10-formyltetrahydrofolate (10-CHO-THF) in an ATP dependent reaction catalyzed by 10-formyltetrahydrofolate synthase. 10-CHO-THF is an essential one-carbon unit donor for the *de novo* synthesis of purine nucleotides, requiring 2 moles of 10-CHO-THF per mole of purine ring formed.

[reviewed in Appling 1991; Depeint *et al.,* 2006]. A supply of mitochondrial produced formate is not an indispensable carbon source for the synthesis of purine nucleotides, however, in murine fibroblasts where mitochondrial production of formate is blocked, a supplement of formate was demonstrated to stimulate cell proliferation two-fold. This indicates the importance of mitochondrial formate as a one-carbon unit donor [Patel *et al.,* 2003]. The role of mitochondrial formate was further elucidated using radioactive labeled variants of formate and serine. Labeled one-carbon units could be traced into both RNA and DNA and their origin could be traced to originate from serine imported to the mitochondria and exported as formate [Patel *et al.,* 2003; Fu *et al.,* 2001].

The mitochondrial permeability transition pore (PT pore) allows, in its open state, diffusion of molecules with molecular masses less than 1.5 kDA. The exact molecular composition of the PT pore remains uncertain, although, both the adenine nucleotide translocase and cyclophilin D are believed to be key structural components. [Reviewed in Javadov and Karmazyn 2007]. Opening of the PT pore has several consequences for the mitochondria, one of which is a loss of mitochondrial inner membrane potential [Zamzami *et al.,* 2005]. Furthermore, it has been demonstrated that opening of the PT pore mediates a release of mitochondrial NAD+ to be hydrolyzed by a NAD+ glycohydrolase, localized outside the matrix, resulting in depletion of mitochondrial NAD+ [Di Lisa *et al.,* 2001]. Opening of the PT pore is regulated by several factors including Ca^{2+}, matrix pH and, most importantly in this context, a depolarization of mitochondrial potential [Bernadi 1992; Bernadi *et al.,* 1994].

The mitochondrial conversion of 5,10-methyleneTHF into 5,10-methenyltetrahydrofolate is a NAD+ dependent reaction and is, furthermore, suggested to be sensitive to the ATP/ADP ratio in the matrix. It is, therefore, conceivable that mitochondrial dysfunction leading to decreased ATP levels and/or a premature opening of the PT pore can result in inhibition of formate produced in mitochondria, which inhibits the *de novo* synthesis of purine nucleotides and deoxyribonucleotides. By comparing human osteosarcoma cells devoid of mtDNA with their parental mtDNA containing cells, it was found that both cell lines grew equally well in complete and glycine-deficient media, demonstrating that the cells without mtDNA were not glycine auxotrophs [Patel *et al.,* 2003]. In the previously described case of a complete inhibition of mitochondrial formate production, cell growth was a two-fold slower [Patel et al 2003]. It is possible that a partial inhibition of mitochondrial formate synthesis would have less dramatic effect on the growth rate, and it would be very interesting to determine whether an addition of glycine could suppress the dNTP imbalance of cells with mitochondrial dysfunctions.

Mitochondrial production of nitric oxide

Nitric oxide (NO) is an uncharged and highly diffusible inorganic signal molecule with a wide variety of roles in the organism. Mitochondrial nitric oxide synthase (mtNOS) is a constitutively expressed enzyme that generates NO in a Ca^{2+} dependent reaction. Furthermore, judging from proven properties of other nitric oxide synthases, production of NO by mtNOS is likely regulated by acetylation and phosphorylation of the enzyme [reviewed in Haynes *et al.,* 2004]. The

enzyme is located in the inner membrane of the mitochondria, [Tatoyan and Giulivi 1998]. Within the mitochondrial membrane, mtNOS physically interacts with both complex I and IV of the ETC [Persichini *et al.,* 2005; Franco *et al.,* 2006]. NO has at sub-micromolar concentrations been demonstrated to act as a competitive inhibitor of complex IV and to inhibit electron transport at complex III. Furthermore, complex I is inhibited after long-term exposure to NO [Cleeter *et al.,* 1994; Poderosa *et al.,* 1996; Riobó *et al.,* 2001] As a result of the inhibitory effects of NO on different sites of the ETC, both the activities of DHODHase and ATP synthase have been demonstrated to be inhibited in response to increased NO levels [Beuneu *et al.,* 2000; Brookes *et al.,* 1999]. Both the localization of mtNOS and the inhibitory effects of NO on the production of ATP by oxidative phosphorylation have lead to the general belief that the purpose of mtNOS is to regulate the oxygen uptake according to the energy requirement of the cell in a short-term fashion.

Nitric oxide produced by mtNOS is most likely not constricted to the mitochondria, but may serve a role in the cytosolic compartment. Efflux of NO from the mitochondria to the cytosol has been demonstrated. This efflux is correlated with the membrane potential of the mitochondria and has been hypothesized to function as a mitochondrial signal to the cytosol, reporting on the energy status of the mitochondria [Boveris *et al.,* 2006; Valdez 2006]. By using mitochondria extracted from different rat tissue, the fraction of cytosolic NO resulting from a mitochondrial efflux was calculated to range from 61% in heart tissue to 18% in brain tissue [Boveris *et al.,* 2006]. Efflux of NO from the mitochondria was demonstrated to be maximal after treatment with oligomycin

and minimal after treatment of CCCP. Treatment with oligomycin inhibits the ATP synthase and produces a hyper polarization of the mitochondrial membrane. Conversely, treatment with CCCP uncouples the membrane. This led to the hypothesis that mtNOS is a voltage dependent enzyme, whose activity is regulated by mitochondrial membrane potential [Boveris *et al.,* 2006; Valdez 2006]. Very little is known about any possible deregulation of mtNOS resulting from mitochondrial dysfunction. Judging from mtNOS activity after treatment with CCCP and oligomycin it can be assumed that a mitochondrial dysfunction that primarily causes an uncoupling of the membrane potential leads to an increased activity while a disruption of the ETC leads to a decreased activity of mtNOS. However, in rats exposed to hypoxia, mitochondria extracted from liver and brain displayed a significantly higher activity of mtNOS than mitochondria from animals subjected to normoxic conditions [Lacza *et al.,* 2001]. This could indicate that the regulation of mtNOS caused by a mitochondrial dysfunction might prove to be more complex.

The role of an efflux of NO from the mitochondria into the cytosol is unknown, however, it has been suggested that it may serve a regulatory role in cell metabolism and proliferation [Boveris *et al.,* 1999; Boveris *et al.,* 2006]. In this case an imbalance of the dNTP pool demonstrated in cells with a mitochondrial dysfunction could be caused by a resulting error in this pleiotropic regulation of cell metabolism. However, NO have several targets in the cellular compartment, and depending on the amount of NO emanating from the mitochondria, these could provide a more direct relationship between the mitochondria and dNTP balance. During the *de novo* pathway the reduction of ribonucleotides to 2'-

deoxyribonucleotides is catalyzed by the rate limiting enzyme ribonucleotide reductase. The reductase constitutes the major regulator of the de novo pathway [Jordan and Reichard, 1998] mediating a reduction of the 2'-hydroxy group in the ribose of the ribonucleotides [Larsson and Reichard, 1966]. The resulting deoxyribonucleotides dADP, dCDP and dGDP undergo phosphorylation yielding the corresponding dNTPs; however dTTP is synthesized from dCDP and dUDP and requires additional steps. Ribonucleotide reductase has been demonstrated to be directly inhibited by NO resulting in a depletion of dNTP proportional to the amount of NO. Treatment of a human lymphoblastoid cell line with different NO prodrugs induced a dNTP imbalance that was comparable to the dNTP profile after treatment with the ribonucleotide reductase inhbitor hydroxy urea [Roy *et al.,* 2004]. Inhibition of the reductase by NO was demonstrated to induce a decrease of dATP and dCTP levels, whereas the dTTP levels were transiently increased [Roy *et al.,* 2004].

The link between mitochondria and dNTP regulation in a non-pathological context

It is possible that the link between mitochondria and dNTP regulation can serve a biological purpose in cells with functional mitochondria in a non-pathological context. Several hypotheses can be made regarding the nature of this link, but we hypothesize that this link is most likely in effect during the progress of an apoptotic event. Mitochondria have an essential role in the progression of apoptotic signals mediated by the released mitochondrial proteins that trigger activation of caspase family proteases or disruption of the electron transport and

mitochondrial membrane potential. Further progress of apoptosis is characterized by chromatin condensation, fragmentation of nuclear DNA, breakdown of the nuclear membrane, externalization of phosphatidyl serine and formation of apoptotic bodies [reviewed in Green and Reed 1998; Wang 2001]. Certain aspects of a disruption of the mitochondrial inner membrane potential in the course of an apoptotic event, is comparable with mitochondrial dysfunctions affecting the transmembrane potential. In the case of an apoptotic event, loss of transmembrane potential has been suggested to be caused by caspase-mediated cleavage of an essential subunit of complex I [Ricci *et al.,* 2004]. Furthermore the existence of a PT pore has been suggested to cause loss of transmembrane potential [Zamzami *et al.,* 2005]. Interestingly, it has been demonstrated that the depletion of mtDNA in human leukaemic lymphoblasts increase resistance to the apoptosis inducing agent vinblastine. The increase was suggested to be caused by a disruption of the ETC, rather than the resulting lower levels of ATP, since an inhibition of the ATP synthase, in mtDNA containing cells, did not increase resistance to vinblastine. This indicates that the mitochondrial inner membrane potential is important for the progression of an apoptotic event and demonstrates a correlation between the fidelity of mtDNA, the function of the ETC and the progress of apoptosis [Jia *et al.,* 1997].

An imbalance of the dNTP pool has been suggested to serve as an initiating factor in the pathway of apoptosis, indicated by an increase of apoptotic events in different cell lines after inhibition of dNTP synthesis and alteration of dNTP levels [Miyashita and Reed 1992; Oliver *et al.,* 1993]. Accordingly, treatment of hemopoietic BAF3 cells with either of the inhibitors hydroxyurea or

methotrexate was demonstrated to induce apoptosis. The inhibitory effects of hydroxyurea and methotrexate are directed at the *de novo* synthesis of dNTP. The increased number of apoptotic events resulting from an inhibition of the *de novo* pathway could be normalized by an addition of deoxyribonucleosides, and thereby supplying the salvage pathway with precursors for adequate dNTP synthesis [Oliver *et al.,* 1996]. During an apoptotic event, loss of transmembrane potential has been demonstrated to be important for the continuation of the process. It is possible that the loss of potential have an inhibitory effect on both the activity of DHODHase and the production of mitochondrial formate as described previously. Hypothetically, by inhibiting the *de novo* synthesis of purine and pyrimidine nucleotides and deoxyribonucleotides, this could promote replication fork arrest and DNA breakage and thereby assist the fragmentation of DNA, defining a role for a link between mitochondrial function and regulation of dNTP pools in the course of an apoptotic event.

Discussion

In previous work, we have demonstrated how depletion of the mitochondrial genome, in two different human cancer cell lines, results in decreased levels and imbalance of cellular dNTP pool [Desler *et al.,* 2007]. This suggests a connection between mitochondrial dysfunction and an erratic regulation of the cytosolic dNTP levels. We argue that in dividing cells, mitochondrial dysfunction is unlikely to cause cytosolic dNTP imbalance by impaired export or excessive import of deoxyribonucleotides. In this review, we describe three candidate pathways, whereby mitochondrial dysfunction can cause depolarization of the

mitochondrial membrane and consequently affect the regulation of cytosolic dNTP levels. These three pathways encompass (1) reduced activity of DHODHase resulting in decreased production of UMP and, thereby, reduction of pyrimidine nucleotides, (2) reduced production and export of mitochondrial one carbon units in the form of formate, resulting in reduced production of purine nucleotides, and (3) erratic regulation of NO produced in mitochondria, affecting both intra mitochondrial and cytosolic regulatory pathways of dNTP synthesis. Even though these three pathways are the most likely mediators of the link between mitochondria and regulation of cytosolic dNTP levels, more experimental data is needed to elucidate this relationship. However, it can not be excluded that the connection is a result of an yet unidentified regulatory pathway within the mitochondria [Desler *et al*., Submitted].

Imbalanced dNTP pools can cause genetic instability in the nuclear genome and in accordance, we have demonstrated the co-occurrence of dNTP imbalance and a increased frequency of chromosomal instability in cell lines devoid of mtDNA [Desler *et al.,* 2007]. Supporting our results, other researchers showed that mitochondrial dysfunction leads to decreased stability of the nuclear genome. A human cervical cancer cell line depleted of mtDNA was reported to be defective in repair of oxidative DNA damage [Delsite *et al.,* 2003]. Chromosomal instability has been demonstrated to occur in human osteosarcoma cell lines devoid of mtDNA. Furthermore, the cells were demonstrated to have tumorigenic properties measured by an increased anchorage independent growth when compared to parental cells with functional mtDNA. It was reported that the reintroduction of functional mitochondria with mtDNA could suppress the

tumorigenic phenotype [Singh *et al.,* 2005]. These observations and other, define a role for dysfunctional mitochondria in the progression of cancer. Due to the known mutagenic effects of dNTP imbalance, it will be important to further elucidate the role of the mitochondrial function on the dNTP regulation in the detrimental relationship between dysfunctional mitochondria and cancer.

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Paper I

Desler, C., Munch-Petersen, B. and Rasmussen, L.J. (2006) The role of mitochondrial dNTP levels in cells with reduced TK2 activity. *Nucleosides, nucleotides & nucleic acids*, **25**, 1171-1175.

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THE ROLE OF MITOCHONDRIAL DNTP LEVELS IN CELLS WITH REDUCED TK2 ACTIVITY

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□ *Both the nuclear and mitochondrial DNA (mtDNA) depend on separate balanced pools of dNTPs for correct function of DNA replication and repair of DNA damage. Import of dNTPs from the cytosolic compartment to the mitochondria has been suggested to have the potential of rectifying a mitochondrial dNTP imbalance. Reduced TK2 activity has been demonstrated to result in mitochondrial dNTP imbalance and consequently mutations of mtDNA in non-dividing cells. In this study, the consequences of a reduced thymidine kinase 2 (TK2) activity were measured in proliferating HeLa cells, on both whole-cell as well as mitochondrial dNTP levels. With the exception of increased mitochondrial dCTP level no significant difference was found in cells with reduced TK2 activity. Our results suggest that import of cytosolic dNTPs in mitochondria of proliferating cells can compensate a TK2 induced imbalance of the mitochondrial dNTP pool.*

Keywords Thymidine kinase 2; Mitochondrial dNTP imbalance; dNTP transport

INTRODUCTION

Imbalanced levels of dNTP result in misincorporation of nucleotides into the DNA, frameshift mutations and stalling of replication fork.[1–3] Mitochondrial dNTP pool imbalance has been demonstrated to induce deletions and depletions in mtDNA,[4] which may cause a number of mitochondrial related diseases such as mitochondrial neurogastrointestinal encephalomyopathy (MNGIE) and mtDNA depletion syndrome (MDS).[4,5] Among the possible mediators of a mtDNA imbalance are dysfunctions of either of the mitochondrial deoxyribonucleoside kinases: thymidine kinase 2 (TK2) or deoxyguanosine kinase (dGK).[1,6]

Recently, the importance of dNTPs from the cytosol to the mitochondria has been demonstrated.[7] Once putative function of this transport is to maintain the balance of the mitochondrial dNTP pool. In nondividing tissue

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though, loss of function mutations in either TK2 or dGK have been demonstrated to result in a mitochondrial dNTP imbalance and the consequential deletions and depletions of mtDNA,[1,6] indicating that import of dNTP to the mitochondria is insufficient in these cells. The cytosolic dNTP levels in proliferating cells are however approximately 10-fold higher compared to the levels of nondividing cells.[8] The increased concentration of cytosolic dNTP could allow a putative import of dNTP to the mitochondria from the cytosol, which would contribute to the maintenance of the mitochondrial dNTP balance in dividing cells.

In the present study, we have modelled a loss of function mutation in TK2 by generating a cell line that can be induced to express lower levels of TK2, and we have measured both the cytosolic and mitochondrial dNTP levels in these proliferating cells.

MATERIALS AND METHODS

Transfection of conditional TK2 antisense plasmids: The doxycyline-responsive (Tet-On) vector pTRE2 (Clontech, Medinova Scientific A/S, Denmark) was used to construct a plasmid expressing the TK2 cDNA in antisense orientation. HeLa cells were transfected with the plasmid using Lipofectamine 2000 (Invitrogen, A/S, Denmark), according to manufacturer's instructions. As selectable marker the plasmid has the puromycin resistance gene and stable clones were selected by incubation in culture medium containing 1 μg/ml puromycin for 10 days. Subsequently, the cells where trypsinated and transferred to 24-well microtiter plates at a density of 50 cells per well. The cells were further incubated 10 days in culture medium without puromycin and finally 7 days in culture medium containing 1 μg/ml puromycin. At each treatment with puromycin, a control culture containing HeLa Tet-On cells not transfected with plasmid, was grown at similar conditions. Before the puromycin treatment was ended, it was made sure that all cells in the control culture had succumbed to the treatment.

Thymidine kinase assay: The activity of TK2 was assayed by its unique ability to phosphorylate thymine-β-D-arabinoside (Ara-T). TK2 phosphorylates AraT with 30–50% of its substrate specificity toward thymidine.[9] Stably transfected HeLa cells was screened by growing cells for 1 week both with and without doxycyline present the in growth media and comparing the TK2 activity of the 2 as described in the following. Cells where lysed by sonication and cell extracts were assayed by adding 15 μl cell extract to a final volume of 50 μl reaction mixture containing: 50 mM Tris-HCl pH 7.5, 10 mM DTT, 2.5 mM ATP, 2.5 mM MgCl, 3 mg/ml BSA, 6 mM NaF, 0.5 mM Chaps and 20 mM [^{3}H] AraT (3 ci/mmol, Morevek Biochemicals, Inc., Brea, CA, USA). The mix was incubated at 37°C, and 10 μl aliquots were removed from the solution at 4, 8 12, and 16 minutes after reaction start, and

spotted onto discs of Whatman DE81 filters. The filters were dried, washed (3 × 10 minutes) with ammonium formate and 5 minutes with autoclaved MilliQ water. Finally the filters were eluted in 500 μl of 0.1 M HCl and 0.2 M KCl and the radioactivity quantified by scintillation counting.

dNTP determination: The whole-cell and mitochondrial dNTP levels were measured using the method of Pontarin et al.[7] and Sherman and Fyfe.[10]

Statistics: Single classification analysis of variance (ANOVA) was used to test for differences in dNTP levels among the different cell lines. Assumptions of normality were checked by visual inspection prior to ANOVA. When the ANOVA indicated significant differences, Tukeys honestly significant method was used to test for differences between the dNTP pools of individual cell lines.

RESULTS

Screening of HeLa cells expressing TK2 antisense RNA in the presence of doxycycline revealed several clones with reduced TK2 activity. One clone that showed approximately 47% reduction of whole-cell TK2 activity upon treatment with doxycycline for a week was chosen for further analysis (data not shown). This cell line was named HeLa TK2$^-$.

The whole-cell dNTP levels were determined in HeLa TK2$^-$ cells grown in the presence of doxycycline for a week, and compared with isogenic cells grown in absence of doxycycline. As illustrated in Figure 1, the doxycycline induced reduction of TK2 activity did not result in a significant difference in dNTP levels compared to cells with a wild type TK2 activity. This indicates

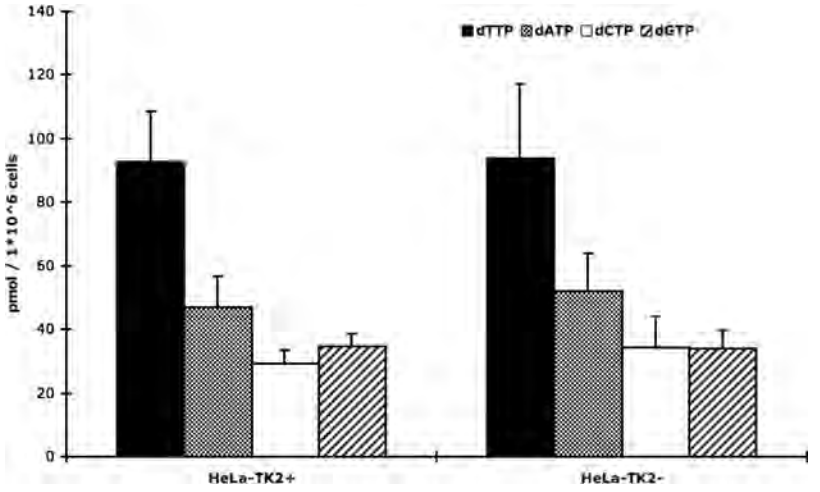

FIGURE 1 The whole-cell levels of dNTP are measured for the dividing cell lines HeLa-TK2$^+$ and HeLa-TK2$^-$ ($n = 12$; error bars indicate SD).

that reduced TK2 activity does not have an effect on the whole-cell dNTP levels.

Similarly, when we determined the mitochondrial dNTP levels of cells grown in the presence as well as the absence of doxycycline, the difference in TK2 activity did not result in a significant difference in the mitochondrial levels of dTTP, dATP and dGTP. The levels of dCTP were significantly higher with an 1.4-fold increase in cells with a reduced TK2 activity (Tukey; $n = 6$; $p < 0.001$) (Figure 2).

DISCUSSION

Even subtle impairments of the TK2 activity have been reported to have an effect on the balance of the mitochondrial dNTP pool. A His-121 to Asn substitution in human TK2 resulted in vitro in a normal activity with dThd as substrate but a 70% reduced activity with dCyd.[11] Furthermore nonreplicating fibroblast cell lines homozygous for the His-121 to Asn TK2 mutation showed decreased levels of mitochondrial dNTP content compared to control cell lines.[1] Our results indicate that a mitochondrial dNTP imbalance is rectified in dividing cells by an import of dNTP from the cytosolic pool. This confirms the initial theory of the potential of such a transport brought forth by both Pontairn et al.[7] and Saada et al.[1]

The mitochondrial levels of dTTP, dATP, and dGTP are not altered by a reduced activity of TK2. If the two salvage kinases TK2 and dGK were the sole providers of mitochondrial dNTP, reduced levels of dCTP and especially dTTP would be expected as a result of reduced TK2 activity. Since no other mitochondrial mediators of dNTP synthesis have been identified,

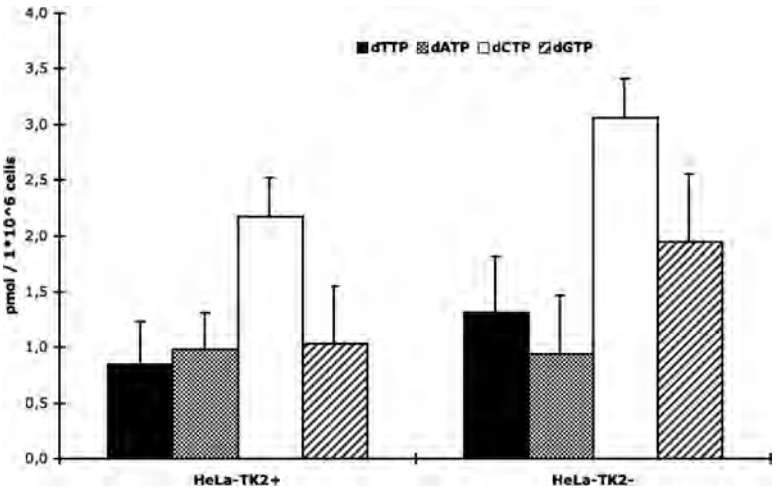

FIGURE 2 The mitochondrial levels of dNTP are measured for the dividing cell lines HeLa-TK2$^+$ and HeLa-TK2$^-$ ($n = 6$; errorbars indicate SD).

an import of dNTP is the most likely explanation for the normal levels of dTTP, dATP, and dGTP observed in the TK2$^-$ cell line.

The significant increase of dCTP levels in response to a reduced TK2 activity is intriguing however the biological explanation and significance of this remains unclear as TK2 also phosphorylates deoxycytidine.

A reduction of TK2 activity did not result in a significant difference between the whole cell levels of dNTP from the levels in cells with wild type TK2 activity. This indicates that the extra contribution needed to sustain the mitochondrial dNTP levels are well within the capacity of the cytosolic de novo and salvage synthesis of dNTP.

The rectifying effect of a dNTP import from the cytosol to the mitochondria in dividing cells serves as an excellent explanation as to why infants harbouring loss of function mutations in proteins governing the mitochondrial dNTP balance, often escape related symptoms until reaching a certain age. At this time many of the cells go into a nondividing state, which results in a 10-fold decrease of the cytosolic dNTP levels. With the decreased import of dNTP from the cytosol, the mitochondrial dNTP levels become imbalanced resulting in accumulating deletions and depletion of mtDNA giving rise to the symptoms of the patients.

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Paper II

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Mitochondria as determinant of nucleotide pools and chromosomal stability

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Abstract

Mitochondrial function plays an important role in multiple human diseases and mutations in the mitochondrial genome have been detected in nearly every type of cancer investigated to date. However, the mechanism underlying the interrelation is unknown. We used human cell lines depleted of mitochondrial DNA as models and analyzed the outcome of mitochondrial dysfunction on major cellular repair activities. We show that the deoxyribonucleoside triphosphate (dNTP) pools are affected, most prominently we detect a 3-fold reduction of the dTTP pool when normalized to the number of cells in S-phase. It is known that imbalanced dNTP pools are mutagenic and in accordance, we show that mitochondrial dysfunction results in chromosomal instability, which can explain its role in tumor development. We did not find any straightforward correlation between ATP levels and dNTP pools in cells with defective mitochondrial activity. Our results suggest that mitochondria are central players in maintaining genomic stability and in controlling essential nuclear processes such as upholding a balanced supply of nucleotides.

Keywords: Mitochondrial disease; Cancer; Chromosomal instability; DNA repair; dNTP levels

1. Introduction

Mitochondria are present in almost all eukaryotic cells and most human cells contain 10^3–10^4 mitochondria, each with several copies of a 16.6 kbp circular mitochondrial DNA molecule (mtDNA) [1]. Several factors can contribute to genomic instability of mtDNA such as increased levels of reactive oxygen species (ROS) that originate from the mitochondrion itself [2] or inactivation of nuclear genes encoding proteins essential for mtDNA maintenance resulting in deletions in and depletion of mtDNA [3–6]. One of the best-characterized functions of mitochondria is the production of ATP by oxidative phosphorylation. Of the 90 subunits comprising the mitochondrial electron transport chain (ETC), 13 are encoded by the mitochondrial genome itself. Dysfunction of mtDNA, therefore, inhibits ATP production and processes dependent on proper function of the ETC [7].

Mutations in mtDNA are associated with cancer as dysfunctional mitochondria have been reported in a variety of cancers including ovarian, thyroid, salivary,

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kidney, liver, lung, colon, gastric, brain, bladder, head and neck, and breast cancers [8–10]. It was recently demonstrated that the invasive phenotype of human cells depleted of mtDNA (ρ^0) could be reversed by introducing exogenous wildtype mitochondria (ρ^+) [11–13]. Along these lines, it has been demonstrated that depletion of mtDNA abolishes androgen dependence of prostate cancer cells, removing the requirement of androgen for cell proliferation [14]. These results suggest that mitochondrial dysfunction contributes to development of tumors. The molecular mechanism underlying the association between mitochondrial dysfunction and tumorigenesis is largely unknown; however, our earlier reports suggest that nuclear DNA repair pathways are implicated. We have shown that human cells depleted of mtDNA (ρ^0 cells) have reduced repair of oxidative DNA damage, while yeast cells depleted of mtDNA have increased mutation frequencies in the nuclear genome [15,16]. The mitochondria-mediated mutator phenotype detected in yeast ρ^0 cells can be suppressed by inactivating subunits of the error-prone DNA repair (Rev1, Rev3, and Rev7). Error-prone repair is involved in bypass of several types of DNA lesions that have the potential to inhibit chromosome replication. In error-prone translesion synthesis (TLS), a non-replicative DNA polymerase replaces the replicative DNA polymerases when these stall at DNA lesions in the template strand [17,18]. Therefore, one interpretation of our previous findings is that mitochondrial dysfunction limits or decreases nuclear DNA repair resulting in unrepaired DNA lesions, which are subsequently converted into mutations by TLS. Such an increase in unrepaired DNA lesions could be a result of impaired function of one or several of the major cellular repair pathways such as nucleotide excision repair (NER), base excision repair (BER), mismatch repair (MMR) and/or recombinational repair (RER) [19]. Another explanation for the mutator phenotype observed in ρ^0 cells [15] is that mitochondrial dysfunction generates excessive DNA damage that is converted into mutations by TLS. Increased error-prone bypass of DNA lesions has been suggested to be characteristic of imbalanced dNTP pools [20]. In yeast, DNA damage results in increased dNTP levels and this dNTP imbalance improves cell survival, possibly because of more efficient TLS [21]. A balanced supply of dNTP is required for DNA synthesis and is crucial for correct DNA repair and replication [22,23]. Imbalance of the dNTP pools has been shown to induce base substitutions as well as frameshift mutations [20,24]. Furthermore, imbalance of the dNTP pools causes delay of DNA replication fork progression and enhances fragile sites where chromosomes are susceptible to breakage. Consequently, imbalance of the dNTP pools can promote chromosome rearrangement, breakage, and loss [22,25–28].

Irrespective of the origin of the observed DNA mutations they may be involved in activating proto-oncogenes and/or inactivating tumor suppressor genes, leading to genomic instability, which play an important role in development of human cancer [29]. Studies have identified two pathways for carcinogenesis: the suppressor pathway characterized by a significant degree of chromosomal instability (CIN) and the mutator pathway characterized by microsatellite instability (MSI) [30]. The CIN pathway is characterized by gross chromosomal rearrangements and translocations whereas the MIN pathway is characterized by sequence instability like point mutations and small insertion/deletions. The MIN pathway is accompanied by mutational inactivation of MMR genes that is believed to lead to an increased rate of mutations in tumor suppressor genes and oncogenes [31]. MIN and defects in MMR are hallmarks of certain forms of colon cancers such as hereditary nonpolyposis colorectal cancer (HNPCC). Conversely, 85% of colon tumors are estimated to be correlated with a significant degree of CIN as compared to normal cells. Two features suggest that MIN and CIN are complementary and functionally important for cancer development: CIN is not observed in MIN-positive tumors, suggesting functional equivalence, and both MIN and CIN appear to arise early in tumorigenesis. Clearly, CIN may favor tumor progression by enhancing loss of heterozygosity (LOH) at tumor suppressor loci. However, the identity of genes that may be responsible for CIN is not clear, although abnormal mitotic function is likely to be involved [31] and recently mitochondrial dysfunction has been added to the list [12].

In order to further clarify the mechanism underlying mitochondria-mediated genomic instability, we analyzed the status of major nuclear DNA repair activities in human cervical and breast cancer cells depleted of mtDNA (HeLa ρ^0 and MDAMB435 ρ^0 cells, respectively). We measured cytosolic dNTP levels of these ρ^0 cells and show that mitochondrial dysfunction does not affect overall repair capacity when measured as the ability to repair endogenous damage of the nuclear genome. However, our results show that mitochondrial dysfunction contributes to imbalanced nucleotide metabolism and chromosomal rearrangements, which could explain the mutator phenotype of ρ^0 cells. The nature of genomic instability observed in human ρ^0 cells is similar to that of the CIN pathway suggesting that mitochondria-mediated mutagenesis does not involve MMR but rather repair systems that generate breaks or gaps if not functioning. These could include BER and RER activities. Our results

also suggest that mitochondrial function is required to prevent chromosomal translocations and rearrangements, which are associated with a variety of human diseases such as cancer [12].

2. Materials and methods

2.1. Cell cultures

ρ^0 Cell lines where produced from their parental ρ^+ cell lines according to [32,33]. The human HeLa ρ^+, HeLa ρ^0, MDAMB 435 ρ^+ and MDAMB 435 ρ^0 cells were maintained in DMEM with Glutamax (Invitrogen, GIBCO) supplemented with 10% FBS (BioChrom AG), 1% Penicillin/Streptomycin (Invitrogen, GIBCO), 2 mM Na-pyruvate and 4 μg/ml uridine unless otherwise stated. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2 for a minimum of 9 days. Cells were grown in 75 cm^2 flasks and were split when approximately 80% confluent. They were removed by trypsin treatment and washed twice with ice cold PBS. Cell concentration and average cell volume were determined in a Z2 Coulter Counter (Beckham Coulter).

2.2. Measurement of ATP levels

ATP levels were determined using the ATP reporter kit from Molecular Probes according to manufacturers instructions. The luminance of the luciferase-based reporter was measured in a Synergy HT multidetection microplate reader (Bio-Tek).

2.3. Measurement of dNTP levels

The DNA polymerase assay is based on the assay originally designed by Solter and Handschumacher [34] and later improved by [35]. In brief, 4×10^6 cells were centrifuged at $750 \times g$ for 5 min and resuspended in 1 ml 60% methanol. The suspension was incubated at −20 °C for 90 min, placed in a heating block and boiled at 100 °C for 3 min in order to remove remaining enzyme activity. The suspension was centrifuged at $17{,}000 \times g$ for 15 min and the supernatant slowly frozen in liquid nitrogen. Methanol was evaporated overnight using a vacuum pump and the residue was rehydrated in 300 μl Ultra-pure H_2O (Invitrogen, GIBCO). Reaction mixtures (total volume of 100 μl) contained 15 μl cell extract, 50 mM Tris pH 8, 5 mM $MgCl_2$, 10 mM DTT, 1 unit Klenow polymerase (Fermentas), 5 μM template oligonucleotide, and 0.48 μM [^{3}H]-dATP (21 Ci/mmol (Vitrax) for dTTP, dCTP and dGTP determinations) or 0.11 μM [^{3}H]-dTTP (87 Ci/mmol (Perkin-Elmer) for dATP determination). The solution was incubated for 30 min at 37 °C. Three 10 μl aliquots were spotted onto Whatman DE81 paper discs, dried, washed (3 × 10 min) with 5% Na_2HPO_4, and rinsed once with sterile water. The papers were transferred to scintillation tubes and 0.5 ml of eluent (0.1 M HCl and 0.2 M KCl) was added. The tubes were shaked for 30 min and 2.5 ml of Eco-Scint was added. Radioactivity was measured in a 1219 Rackbeta liquid scintillation counter (WALLAC).

2.4. Thymidine Kinase 1 (TK1) activity determination

1×10^6 cells were centrifuged at $1400 \times g$ for 5 min and resuspended in 100 μl extraction buffer (50 mM Tris–HCl pH 7.5, 2 mM DTT, 5 mM Benzamidin, 0.5 mM PMSF, 50 mM E-amino caproic acid, 5 mM EDTA, 10% Glycerol and 0.1% Triton X-100). The suspension was homogenized by 3 × 5 s sonication on ice with 1 min intervals. The homogenate was examined microscopically to ensure complete breakage of cell membranes and centrifuged at $15{,}000 \times g$ for 10 min. The activity of TK1 was assayed by its unique ability to phosphorylate 3′-azido-2′,3′-deoxythymidine (AZT) [36]. One microlitre of the supernatant was mixed with an assay mix solution to a final volume of 50 μl (50 mM Tris–HCl pH 7.5, 10 mM DTT, 2.5 mM ATP, 2.5 mM $MgCl_2$, 3 mg/ml BSA, 6 mM NaF, 0.5 mM Chaps and 15 μM [^{3}H]-AZT (1.8 Ci/mmol)). The assay mix was incubated at 37 °C. At 4, 8, 12 and 16 min after reaction start, 10 μl aliquots were removed from the solution and spotted onto Whatman DE81 paper discs. The filters were dried, washed with 5 mM ammonium formate (3 × 10 min) and once with autoclaved MilliQ water. The nucleotides were eluted by shaking with 0.5 ml eluent (0.1 M HCl and 0.2 M KCl) for 30 min, whereafter 2.5 ml of Eco-Scint (National Diagnostics) was added. Radioactivity was measured in a 1219 Rackbeta liquid scintillation counter (WALLAC).

The protein concentration of each cell extract was determined by Bradford assay.

2.5. Cell cycle analysis with a fluorescence-activated cell sorter

For cell cycle analysis, cells were prepared and stained using the Absloute-S kit (Phoenix Flow Systems) according to manufacturers instructions. The kit utilizes BrdU incorporation to determine the number of cells in S-phase. DNA content was measured using a Becton Dickinson FACSCalibur fluorescence-activated cell sorter (FACS), Cell QuestPro software and WinList software.

2.6. Comet assay

A modified version [37] of single-cell gel electrophoresis was used to measure DNA lesions resulting from endogenous DNA damage. The modifications include reduced electrophoresis (10 min at 300 mA) and the use of a Dialux 22EB (Leica) microscope and Comet Assay III (Perceptive instruments) software to analyze assayed cells and calculate comet tail moment. Mean Tail Moment was calculated from the comet tail moment of 50 randomly selected cells and each data point consists of a triplicate of Mean Tail Moments. This value was then used as a quantitative index of DNA breaks.

2.7. Microsatellite instability (MSI) analysis

Cell lines were carried through approximately 25 passages where after chromosomal DNA was extracted from

1 to 2×10^6 cells using the Genelute kit (Sigma) according to manufacturers guidelines. Microsatellite analysis was performed as previously described [38] using the HNPCC Microsatellite Instability Test kit according to manufacturers instructions (Roche). Analyzed microsatellite loci included D5S346 (APC), BAT25, BAT26, D17S250 (Mfd15CA) and D2S123. As starting material we used 100 ng genomic DNA and PCR products were analyzed on an ABI 377 sequence analyzer, and GeneScan 3.0 (Perkin-Elmer).

2.8. Incision repair assay

Oligonucleotides were purchased from DNA Technology (Aarhus, Denmark) and 5′end-labeled using T4 polynucleotide kinase (MBI, Fermentas) and (γ^{32}P)ATP (Amersham Biosciences). Unincorporated radioactivity was removed with G25 spin columns.

Isolation of mitochondria was accomplished using a combination of differential centrifugation and Percoll gradient centrifugation employing a protocol modified from [39]. Briefly, actively growing cells were washed once with ice cold PBS, pelleted and resuspended in M-SHE buffer (0.21 M mannitol, 0.07 M sucrose, 10 mM Hepes (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.15 mM spermine, 0.75 mM spermidine, 5 mM DTT, 2 μM PMSF, 2 μg/ml Protease inhibitor cocktail Set III (CalBiochem)). The cell suspension was homogenized using a teflon-to-glass homogenizer and the mitochondrial fraction was separated from the nuclear pellet. The supernatant containing the mitochondria was subjected to differential and Percoll gradient centrifugation and protein concentration was determined by the Lowry method. Purified mitochondria were stored as pellets at −80 °C. Nuclear extracts were prepared in parallel with the mitochondrial isolation, according to modified versions of two protocols [40,41]. Briefly, the nuclear pellet (obtained during preparation of mitochondria) was treated with 10 mM Hepes (pH 7.9), 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA (pH 8.0), 0.5 mM PMSF, 20% glycerol, 0.1% NP40 and 2 μg/ml Protease inhibitor cocktail Set III (CalBiochem) and subsequently subjected to centrifugation. The supernatant was dialyzed against 50 vol.% of a buffer containing 20 mM Hepes pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF. Protein concentration was determined by the Lowry method and the purified nuclei were stored in aliquots at −80 °C. Glycosylase/AP-lyase activity in nuclei and mitochondria was measured using an oligonucleotide incision assay as previously described [42]. Briefly, to disrupt intact mitochondria, these were resuspended in a buffer containing 20 mM HEPES-KOH (pH 7.6), 1 mM EDTA, 2 mM DTT, 300 mM KCl, 5% glycerol, and 0.05% Triton X-100. The mitochondrial suspension was subsequently diluted to a final concentration of 5 μg/μl protein and 100 mM KCl. Nuclear and mitochondrial incision reactions (20 μl) contained 20 mM HEPES-KOH (pH 7.6), 5 mM EDTA, 5 mM DTT, 75 mM KCl, 5% glycerol, 0.1 mg/ml BSA, and 90 fmol ^{32}P-labeled duplex oligonucleotide. Reaction mixtures were incubated at 37 °C for 1 h and then terminated by adding proteinase K and SDS to a final concentration of 0.2 mg/ml and 0.4%, respectively, and incubated for 15 min at 55 °C. FA-loading dye (80% formamide, 10 mM EDTA (pH 8.0)) was added to the samples, heated for 2 min at 95 °C and analyzed on a 20% denaturing polyacrylamide gel containing 7 M urea. The radioactively labeled DNA was visualized using a Personal Molecular Imager® (BioRad) and quantified using Quantity One software (BioRad). Incision activity was calculated as the ratio of damage-specific cleavage product to the total product and substrate in the reaction.

2.9. Chromosomal instability (CIN) assay

CIN assay was performed by Spectral Karyotyping analysis (SKY). After mitotic arrest for 2 h with Colcemid, cells were harvested and treated with hypotonic solution according to the standard protocol. Chromosome slides were prepared using air-drying. After sequential digestion with RNAse and pepsin according to the procedure recommended by Applied Spectral Imaging, Inc. (ASI: Carlsbad, CA), the chromosomes were denatured in 70% formamide and hybridized with human SKY paint probes tagged with various nucleotide analogues (i.e., a mixture of individual chromosomal DNAs prepared by flow-sorting and PCR amplification) [12]. The multiple fluorescence color images of chromosomes generated by Rhodamine, Texas-Red, Cy5, FITC and Cy5.5 were captured using a Nikon microscope equipped with a Spectral cube and Interferometer module and analyzed using SKY View software (Version 1.62). Chromosome number and chromosomal rearrangements or alterations including simple balanced translocation or unbalanced (or non-reciprocal) translocation, deletion and duplication, were analyzed to determine the lineage of individual knock-out cell lines, compared to the original wild-type counterpart.

2.10. Statistical analysis

Single classification analysis of variance (ANOVA) was used to test differences in dNTP pools and ATP levels between cell lines. Assumptions of normality were checked by visual inspection prior to ANOVA. When the ANOVA indicated significant differences among the cell lines, Tukeys honestly significant method was used to test for differences between dNTP pools of individual cell lines.

3. Results

3.1. Depletion of mtDNA leads to reduced levels of ATP

Damage to mtDNA causes reduced activity of the ETC resulting in decreased ATP level, leaving glycolysis as the sole source of ATP supply in human cells. Since ATP is essential for multiple cellular processes, including DNA precursor synthesis and DNA repair activity,

Table 1
ATP levels in human cells with dysfunctional mitochondria

Cell line	ATP concentration (mM)	
	HeLa	MDAMB435
ρ^+	1.44 ± 0.4	2.1 ± 0.2
ρ^0	0.36 ± 0.06	0.13 ± 0.02
ρ^+/ρ^0	~4	~16

The ATP levels of HeLa and MDAMB435 (ρ^+ and ρ^0) cells were determined and ρ^+/ρ^0 reflects the ratio of ATP levels between ρ^+ and ρ^0 cells ($n = 3$).

we compared the level of ATP in ρ^0 cells with the ATP level of their corresponding parental ρ^+ cell lines. Our results show that intracellular ATP levels are decreased 4-fold in HeLa ρ^0 cells and 16-fold in MDAMB435 ρ^0 cells (Table 1), confirming that the intracellular level of ATP is reduced in cells with dysfunctional mitochondria as expected.

3.2. Mitochondrial dysfunction causes imbalanced dNTP pools

We have previously shown that mitochondrial dysfunction causes genomic instability that is due to TLS [15]. Genomic instability involving TLS has been suggested to be caused by imbalanced nucleotide pools [20,21] and therefore, we investigated if the cellular dNTP pools are affected by mitochondrial dysfunction. Using a polymerase extension assay, we measured dNTP pools in two different human ρ^0 cell lines and compared the results to their respective parental ρ^+ cell lines. Within the cell, the dNTP content is separated into two compartments, the cytosolic and the mitochondrial, which provide dNTP for synthesis of the nuclear and mitochondrial DNA, respectively. We found no difference in mitochondrial dNTP levels between ρ^0 cell lines and their corresponding ρ^+ cell lines (data not shown). Since the mitochondrial dNTP content only encompass 1/10 of the total dNTP pool size in proliferating ρ^+ cells (data not shown), we did not exclude the mitochondria from the cytosolic fractions in our dNTP assays. Our results show that there is a significant difference in whole cell dNTP levels between ρ^0 cell lines and their corresponding ρ^+ cell lines (Fig. 1A). In HeLa ρ^0 cells the whole cell dTTP and dCTP levels are reduced 5–6 fold compared to the corresponding pools in the parental ρ^+ cell line (Tukey, $n = 3$; $p < 0.001$). We did not find any convincing difference in dATP and dGTP levels between HeLa ρ^+ and HeLa ρ^0 cells. In order to exclude that reduced dNTP pools in cells with dysfunctional mitochondria is specific for HeLa cells, we also measured dNTP pools in MDAMB435 ρ^0 cells. The dNTP pools were also decreased in these cells, however, the differences were more evenly distributed between the dTTP, dATP, dCTP and dGTP pools with a 2–3 fold decrease compared to MDAMB435 ρ^+ cells (Tukey, $n = 3$; $p < 0.001$) (Fig. 1A).

Cytosolic dNTP levels are reduced in non-proliferating cells compared to proliferating cells due to reduced activity of the ribonucleotide reductase (RNR) and thymidine kinase 1 (TK1) [43]. Cells with dysfunctional mitochondria grow more slowly than ρ^+ cells and cell cycle analysis showed a lower fraction of ρ^0 cells in S-phase compared to ρ^+ cells (Fig. 2). To ensure that the lower dNTP levels in ρ^0 compared to ρ^+ cells are not a result of a smaller number of ρ^0 cells in S-phase, we normalized the dNTP levels to the number of S-phase cells (Fig. 1B). As shown in Fig. 1B, upon normalization a difference in dTTP and dCTP levels between HeLa ρ^0 and ρ^+ cells persisted. Low levels of all four deoxynucleotides were also found in the MDAMB435 ρ^0 cells when compared to the parental ρ^+ cells. These results exclude the difference in cell cycle distribution between ρ^0 and ρ^+ cells as a critical factor for the lowered dNTP levels in cells with dysfunctional mitochondria. We investigated if the whole cell dNTP levels of ρ^0 cell lines could be restored to the levels of the corresponding ρ^+ cell lines by increasing the concentration of uridine supplement. We treated cells with up to 100 μg/ml uridine, but found no significant effect on the dNTP levels of both HeLa and MDAMB435 ρ^0 and ρ^+ cells (data not shown). Our results suggest that mitochondrial dysfunction results in dNTP pool imbalance which is not restorable with addition of uridine.

Salvage of deoxynucleosides is carried out by deoxynucleoside kinases that catalyze transfer of the first phosphate group from a phosphate donor, typically ATP, to the 5′-OH group at the deoxyribose. TK1 is the cytosolic thymidine kinase phosphorylating only thymidine and deoxyuridine [44]. In order to investigate if altered activity of TK1 could explain the low levels of dTTP in ρ^0 cells, we measured TK1 activity by assaying the phosphorylation of [^{3}H]-AZT. We found HeLa ρ^+ and ρ^0 cells to have identical TK1 activities whereas MDAMB435 ρ^+ cells have a TK1 activity twice as high than MDAMB435 ρ^0 cells (Fig. 3). We conclude that decreased dTTP pool in HeLa ρ^0 cells is not caused by altered TK1 activity due to mitochondrial dysfunction. The decreased dNTP levels in MDAMB435 cells are not explainable as a result of the change in TK1 activity since all measured deoxyribonucleotides are affected and TK1 has a very restricted substrate activity.

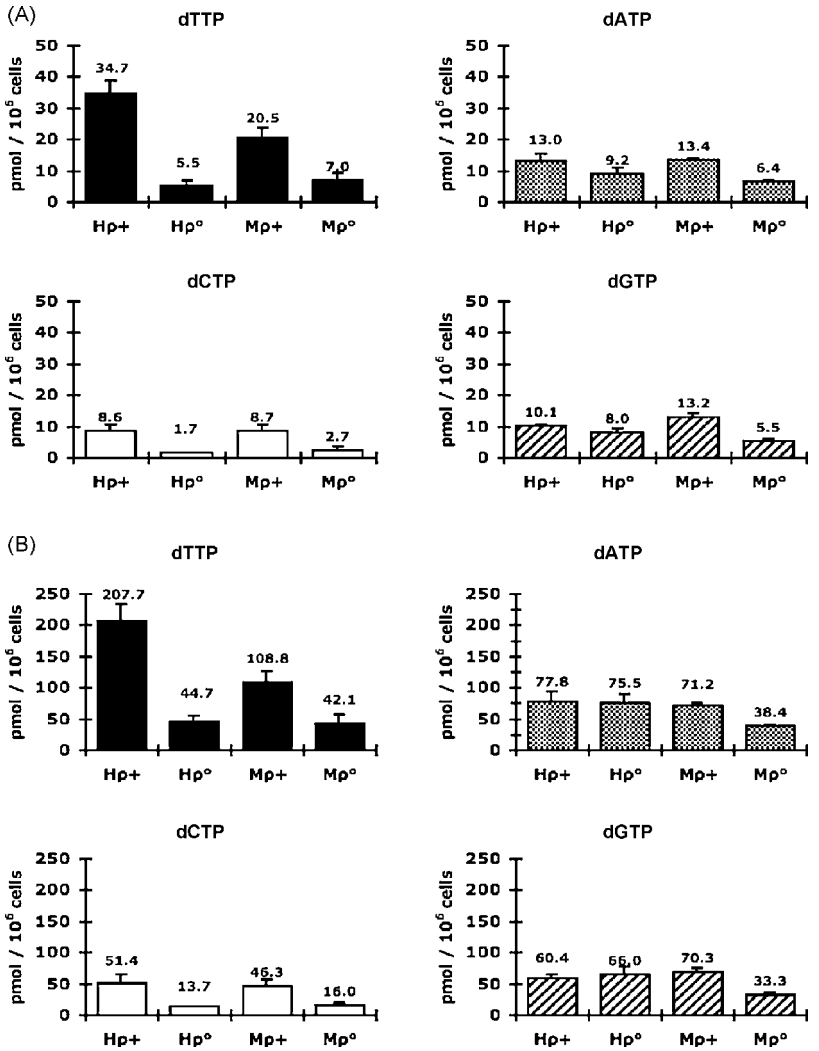

Fig. 1. (A) Nucleotide pools in cell with dysfunctional mitochondria. From top left to bottom right are shown the dTTP, dATP, dCTP and dGTP levels, respectively. (B) Total levels of dNTP were normalized to the number of cells in S-phase in HeLa ρ^0, MDAMB435 ρ^0 and parental ρ^+ cell lines ($n = 3$; error bars indicate S.D.). The abbreviations used are: Hρ^+, HeLa ρ^+; Hρ^0, HeLa ρ^0; Mρ^+, MDAMB435 ρ^+; Mρ^0, MDAMB435 ρ^0.

3.3. Dysfunctional mitochondria and nuclear DNA repair

Imbalanced nucleotide pools are believed to affect integrity of genomes and we have previously demonstrated that cells with dysfunctional mitochondria display a mutator phenotype, which is associated with the Rev1, Rev3 and Rev7 subunits of the error-prone repair pathway [15]. These results could indicate that there is a higher amount of unrepaired endogenous DNA lesions in ρ^0 cells compared to the parental ρ^+ cells. Along these lines, we also showed that human ρ^0 cells contain increased nuclear DNA damage after treatment with hydrogen peroxide [16]. Together this suggests an impairment of nuclear DNA repair activities in response to mitochondrial dysfunction in both yeast and human cells. Therefore, we initiated studies to clarify which of the major nuclear DNA repair activities that could be affected by defective mitochondrial function.

First, we used the COMET assay to achieve a general assessment of the endogenous DNA damage level of human ρ^0 cells. This assay is a rapid and relatively sensitive method for detecting alkali labile sites as well as single and double stranded breaks in the DNA. Alkaline treatment causes the DNA strands to unwind allowing visualization of alkali labile sites, double stranded breaks, single stranded breaks, crosslinks and incom-

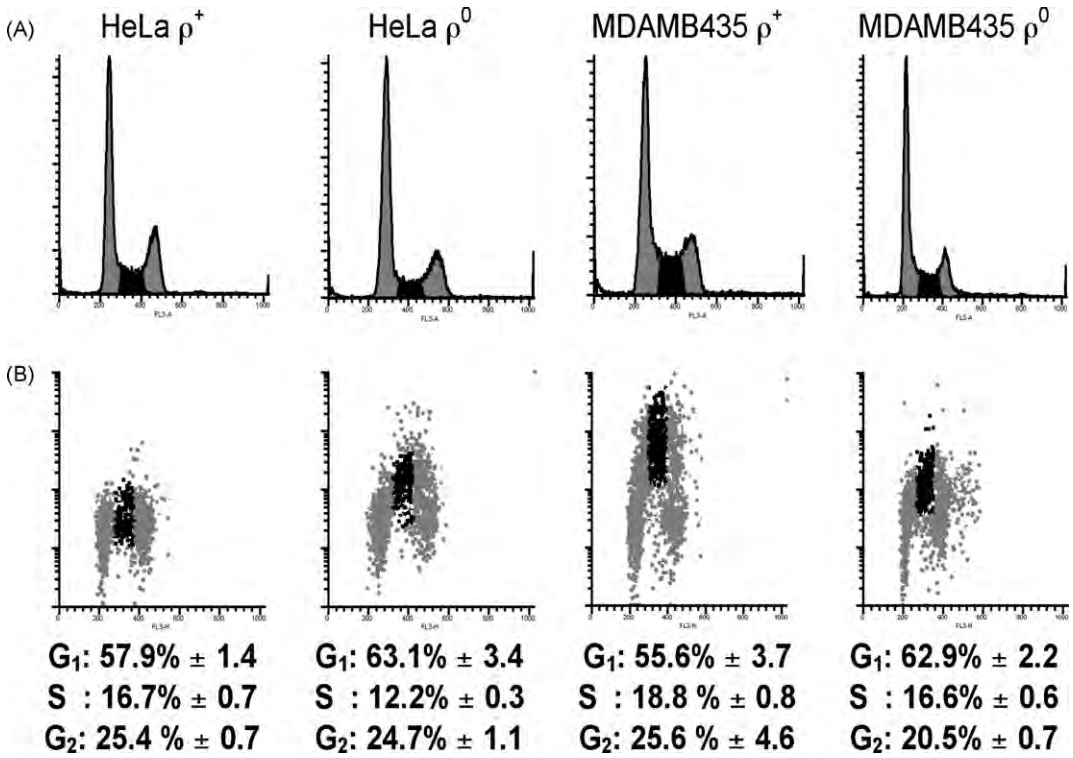

Fig. 2. The cell cycle distribution of HeLa ρ^0, MDAMB435 ρ^0 and parental ρ^+ cell lines, was determined by flow cytometry. Panel A displays the profile obtained by staining with propidium iodide. Panel B displays the staining of BrdU incorporated into DNA. Black dots represent cells in S-phase.

plete excision repair sites, resulting from endogenous DNA damage [37]. These types of DNA damage are generally corrected by the BER, NER and RER pathways. The comet assay gives a rough estimate of whether cells with dysfunctional mitochondria contain increased endogenous nuclear DNA damage compared to wild-type cells. We did not find any significant difference between the HeLa ρ^0 and MDAMB435 ρ^0 cell lines and their parental ρ^+ cell lines using the COMET assay (data not shown). Our data do not exclude a difference in repair ability of endogenous DNA damage between ρ^0 and ρ^+ cells; however, any potential differences are too subtle to be measured by the COMET assay.

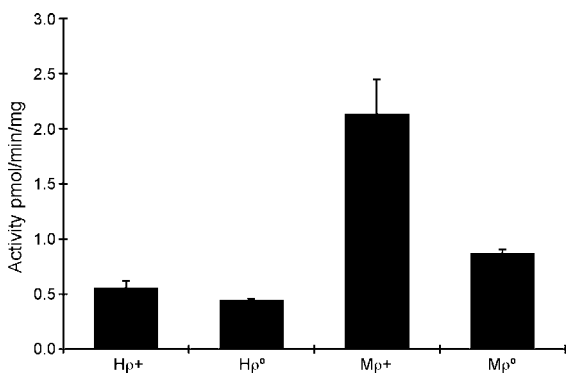

Fig. 3. Thymidine Kinase 1 activity in cells with dysfunctional mitochondria. Thymidine Kinase 1 (TK1) activity was assayed by its selective ability to phosphorylate [^{3}H]-AZT ($n = 2$; error bars indicate S.D.). The abbreviations used are: Hρ^+, HeLa ρ^+; Hρ^0, HeLa ρ^0; Mρ^+, MDAMB435 ρ^+; Mρ^0, MDAMB435 ρ^0.

HNPCC tumors and a proportion of sporadic colon cancers are deficient in MMR activity and these tumors display a widespread genomic instability that is most easily recognized by examining short repeated sequences (microsatellites). MSI is a unique form of genomic instability and is most often observed in cells deficient in either hMSH2 or hMLH1. Defects in MMR lead to genomic instability characterized by expansion or contraction of simple repeat sequences in the nuclear DNA. In order to clarify if the mitochondrial-mediated genomic instability is caused by defects in MMR activity, we investigated the function of MMR in ρ^0 cells by analyzing five microsatellite markers (two mononucleotide repeats: BAT25 and BAT26; and three

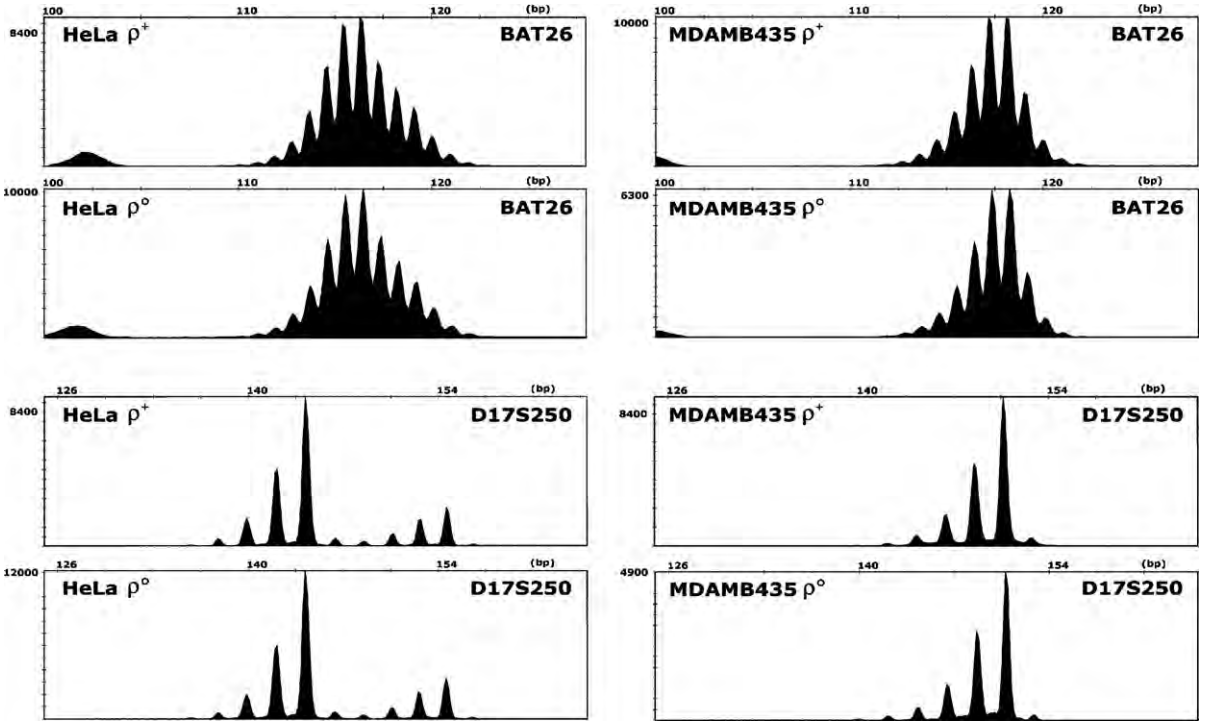

Fig. 4. Microsatellite instability in cells with dysfunctional mitochondria. MSI analysis of HeLa ρ^0, MDAMB435 ρ^0 and parental ρ^+ cell lines. The figure shows selected examples from microsatellite analysis at the BAT26 and D17S250 loci. All loci were assayed in triplicates and appear stable (data not shown).

dinucleotide repeats: D2S123, D5S346 and D17S250) recommended in the National Cancer Institute (NCI) set. We found no microsatellite instability in the NCI set of markers in HeLa ρ^0 and MDAMB435 ρ^0 cells when compared to their parental ρ^+ cells (Fig. 4). Our results suggest that nuclear MMR activity is preserved in cells with defective mitochondrial function.

Thymine depletion is toxic, partly due to an increase in the dUTP/dTTP ratio and subsequent incorporation of dUTP instead of dTTP into DNA. Uracil incorporation in DNA is mutagenic and is usually repaired by BER. The pathway is initiated by removal of the incorrect base by a uracil glycosylase to create an AP site followed by nicking of the damaged DNA strand by AP endonuclease upstream of the AP site allowing excision of the AP site. It has previously been shown that AP endonuclease activity is decreased in ρ^0 cells and that AP endonuclease deficiency results in sensitivity to thymine deprivation [12,45,46]. Since we demonstrate a low level of the dTTP pool in ρ^0 cells, it is possible that the dUTP/dTTP ratio in ρ^0 cells is affected and that the mutator phenotype observed in ρ^0 cells is caused by excess uracil incorporation in DNA. Therefore, we compared incision activities of mitochondrial as well as nuclear extracts in ρ^+ and ρ^0 cells on uracil DNA lesions and oxidized bases (Fig. 5). We did not detect any significant differences in incision activities suggesting that the DNA incision step of BER is unaffected in ρ^0 cells.

3.4. Chromosomal instability

Previous studies demonstrate that imbalanced dNTP pools can lead to delay of replication fork progression, double strand breaks, and expose fragile sites [20,22,24–28]. All these DNA lesions are potential originators of chromosomal instability such as translocations and rearrangements. We therefore carried out SKY analysis to investigate if dysfunctional mitochondria and/or imbalanced dNTP pools caused CIN. SKY analysis revealed specific chromosomal changes in ρ^0 cells. These were translocations at *t*(20;9) and *t*(22;6) (Fig. 6). Interestingly, database analysis of cancer chromosomes (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=cancerchromosomes) indicate that the translocations identified in ρ^0 cell lines are found in many tumors including breast tumors [12]. Our results suggest that mitochondrial dysfunction causes imbalanced dNTP pools, which result in DNA damage that promote chromosomal translocations.

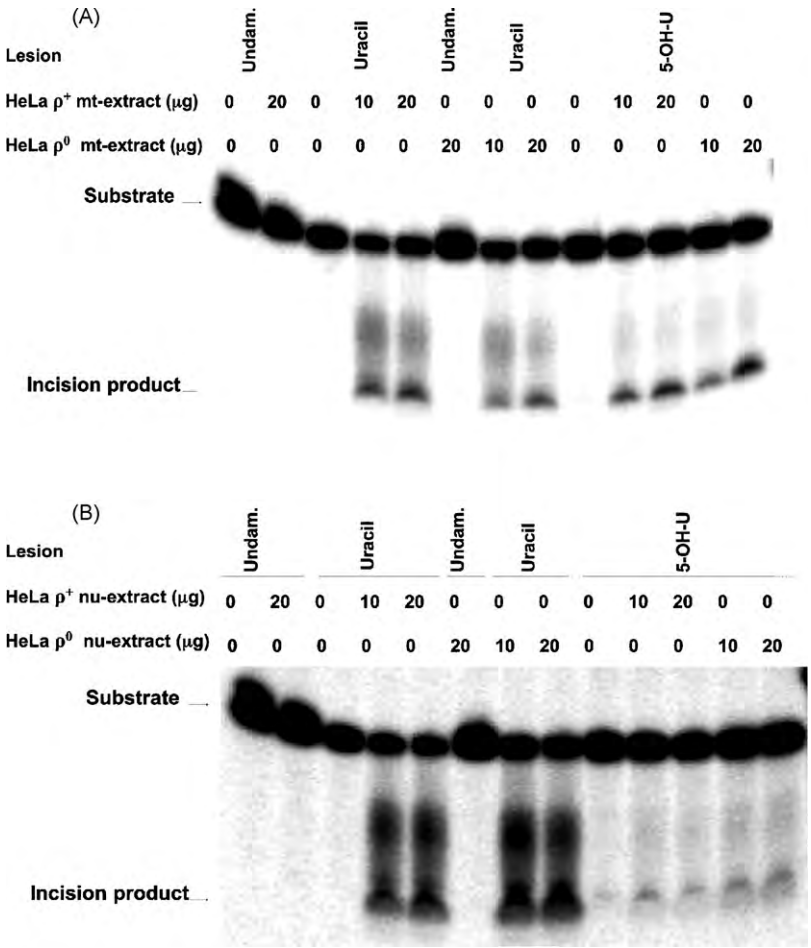

Fig. 5. Base incision capacity in cells with dysfunctional mitochondria. Capacity of (A) mitochondrial and (B) nuclear extracts from HeLa wt and HeLa ρ^0 cells, respectively, to incise oligonucleotide substrates containing a single uracil or 5-OH-uracil lesion. Ninety femtomoles of a 30-nt oligonucleotide with a single uracil or 5-OH-uracil residue was incubated with the indicated amount of extract for 1 h at 37 °C.

4. Discussion

Reported intracellular concentrations of the four dNTPs vary considerably between organisms and cell lines. In addition, there appears to be a consensus that the pools are not equal but can differ up to 10-fold. Generally, the dTTP pool is highest and dGTP the lowest pool in proliferating cells [43,47–53]. In quiescent cells, dNTP levels are several fold lower [49], and in non-proliferating peripheral blood lymphocytes which are truly G_0 cells, the dTTP pool is reported to be several fold lower than the other three pools [43,47,48,54]. Thus, low dTTP pool may explain the finding of the pronounced DNA strand-break repair promoting effect of 1–2 μM thymidine in UV-irradiated non-dividing human lymphocytes [55]. In order to investigate the mechanism underlying mitochondria-mediated genomic instability, we examined the relationship between mitochondrial dysfunction, dNTP levels, DNA repair activity, and chromosomal stability. We show that mitochondrial dysfunction leads to a decrease in dNTP pools as well as genomic instability. The nature of this instability is similar to CIN, which is one of the hallmarks of cancer cells.

Due to the number of mitochondrial-encoded subunits of the ETC, the ETC of ρ^0 cells is non-functional resulting in a reduced ATP production. An insufficient supply of ATP has been suggested to contribute to the mutator phenotype observed in ρ^0 cells by affecting ATP-dependent pathways involved in transcription, DNA replication, DNA repair and DNA recombination [56]. Several processes of dNTP synthesis are ATP-dependent, most notably the phosphorylation of deoxyribonucleoside mono- and diphosphates yielding

Cell	t(1;7)	t(1;10)	t(11;8)	t(13;11)	t(14;1)	t(14;16)	t(18;19)	t(19;6)	t(20;9)	t(20;21)	t(22;6)
Rho+											
1		x	x	x	x	x	x			2x	
2	x	x	x	x	x	x	x	x		2x	
3	x	x		x	x	x	x			2x	
4		x		x	x		x	x		2x	
5	x	x	x	x	x	x	x			2x	
6	x	x		x	x	x	x	x		2x	
7	x	x	x	x	x	x	x	x		2x	
8	x	x	x	x	x	x	x	x		2x	
9	x	x	x	x	x	x	x	x		2x	
10	x	x	x	x	x	x	x	x		2x	
11	x	x	x	x	x	x	x	x		2x	
12	x	x		x	x	x	x	x		2x	
13	x	x		x	x	x	x	x		2x	
14	x	x		x	x	x		x		2x	
15	x	x		x	x	x	x	x		2x	
Cell	t(1;7)	t(1;10)	t(11;8)	t(13;11)	t(14;1)	t(14;16)	t(18;19)	t(19;6)	t(20;9)	t(20;21)	t(22;6)
Rho0											
1	x	x		x		x	x		x	2x	x
2	x	x	x	x	x	x	x	x	x	2x	x
3	x	x	x		x					2x	x
4	x			x	x	x	x	x	x	2x	x
5		x		x	x	x	x		x	2x	x
6	x	x		x	x	x	x	x	x	x	x
7	x	x		x	x	x	x	x	x	x	
8	x	x		x		x	x	x	x	2x	x
9	x	x	x	x	x		x	x	x	2x	x
10	x	x		x	x	x	x	x	x	2x	x
11	x		x	x	x	x	x	x	x	2x	x
12	x	x	x	x	x	x	x		x	2x	x
13	x	x	x	x	x	x	x	x	x	2x	
14	x	x		x	x	x	x	x	x	2x	x
15	x	x		x	x	x	x	x	x	2x	

Fig. 6. Chromosomal instability in cells with dysfunctional mitochondria. Chromosomal instability in parental ρ^+ and ρ^0 MDAMB435 breast cancer cells was analyzed as described in Section 2. Translocations present in both cell lines are presented in gray column whereas specific translocation found in ρ^0 cells are presented in yellow columns. Minimum of 15 metaphases were analyzed in each case. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

dNTP. Therefore, it is possible that the low and imbalanced nucleotide pools characterizing human ρ^0 cells is a result of low ATP levels in these cells. Our results show an expected decrease of ATP levels in both HeLa ρ^0 and MDAMB435 ρ^0 cells compared to their parental ρ^+ cell lines. However, the correlation between the decrease of ATP levels and the decrease of dNTP levels is not identical in the two cell lines. This suggests that factors and pathways, other than ATP synthesis, are compromised and contribute to down regulation of dNTP synthesis. Interestingly, we found that decreased levels of ATP did not result in any reduction of TK1 activity in HeLa cells or impairment of repair of endogenous nuclear DNA damage in both cell lines, indicating that the ATP levels of ρ^0 cells are sufficient for, at least, these processes.

Retrograde communication between mitochondria and the nucleus has been demonstrated and one suggested role of this communication is to provide a link between mitochondrial status and cell cycle regulation [13,56,57]. When damage to mitochondria is persistent, it has been suggested that the retrograde communication becomes irregular and has an unfavorable effect on the cell [56]. In human breast cancer ρ^0 cells, the retrograde communication between the mitochondria and the nucleus has been suggested to result in altered expression of nuclear genes involved in signaling, cellular architecture, metabolism, cell growth, and apoptosis [33]. Using *S. cerevisae* as a model system, it has been shown that mitochondrial dysfunction leads to altered expression of a group of genes [56], which could explain the imbalanced dNTP levels of ρ^0 cells (data not shown). These genes include *CDC21* expressing thymidylate synthase that has an essential role in the *de novo* synthesis of dTTP by mediating the methylation of dUMP yielding dTMP. Human thymidylate synthase is encoded by *TYMS* and several chemotherapeutic agents that target thymidylate synthase are used for treatment of tumors [58,59]. The effect of these agents is to inhibit the thymidylate synthase and thereby

inhibit the formation of dTTP resulting in stagnation of cell growth. When mouse FM3A cells were treated with the chemotherapeutic agent 5-flourodeoxyuridine (5-FU), the thymidylate synthase was inhibited and an imbalance of the dNTP pools was observed. The levels of dTTP and dGTP were strongly reduced, while the dATP pool was increased [59]. Furthermore, it has been shown that Inosine 5′-monophosphate dehydrogenase type 2 (IMPDH2) involved in nucleotide biosynthesis was upregulated in ρ^0 cells. IMPDH2 is a rate-limiting enzyme in *de novo* guanine biosynthesis [13].

In this study, we show that mitochondrial dysfunction does not result in a substantial reduction in overall repair activities when measured as the ability to repair endogenous damage of the nuclear DNA. Remarkably, we show that the first step in BER, the incision of DNA damage, is unaffected in ρ^0 cells whereas it has previously been shown that the downstream AP-endonuclease in BER is down-regulated in ρ^0 cells [12,45]. The result of such deregulation of BER activities could result in excess of mutagenic AP-sites that can be converted into mutations by the TLS pathway [60]. Our results also show that proliferating ρ^0 cells contain imbalanced dNTP pools compared to parental cell lines. Concurrently mitochondrial dysfunction is demonstrated to contribute to chromosomal translocations and rearrangements. The nature of the genomic instability observed in human ρ^0 cells is similar to that of the CIN pathway. This particular form of genomic instability is characterized by mutations in cell-cycle regulators, checkpoint proteins, and structural components of the mitotic spindle [31]. Thus, our results suggest that mitochondrial function is fundamental for maintaining genomic integrity by preventing chromosomal translocations and rearrangements, which are associated with a variety of human diseases such as cancer.

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Paper III

Desler, C., and Rasmussen, L.J.Increased concentrations of dNTP following DNA damage determines the TLS response in *Saccharomyces cerevisiae* Manuscript in preparation

Increased concentrations of dNTP following DNA damage determines the TLS response in *Saccharomyces cerevisiae*

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Abstract

In *Saccharomyces cerevisiae,* DNA lesions that inhibit DNA replication are met by a response in the form of increased S-phase specific dNTP levels. While resulting in a higher mutation frequency, the increase is also essential for cell survival following treatment with DNA damaging agents. Increased levels of dNTP are believed to facilitate DNA translesion synthesis (TLS) at the expense of induced mutations. We propose that different TLS polymerases have their highest activity at different dNTP concentrations, and that the dNTP levels therefore not only facilitate TLS but also regulate the TLS response.

By treating several strains with the alkylating agent methyl-methanesulfonate (MMS), we have shown that dNTP levels increases in logarithmic phase cells in a time-dependent manner. We have demonstrated that after 1 hour of MMS treatment, strains displayed an increase of dNTP levels but no increase in mutation frequencies. After 2.5 hours of MMS treatment strains displayed a further increase in dNTP levels and an increase of mutation frequencies. According to our hypothesis, moderate increased levels of dNTP mediate error-free TLS, while further increase mediates error-prone TLS in response to the DNA damaging agent.

A deletion of *REV3,* encoding the catalytic subunit of the error-prone TLS polymerase ζ, was shown not to induce mutation frequency after 2.5 hours of MMS treatment indicating the involvement of the error-prone polymerase and substantiating our hypothesis.

Using a transcriptional network, we have tried to identify the regulatory pathway responsible for the increase of dNTP concentrations in response to DNA damaging agents. This would allow us to investigate the TLS response in strains with no induced dNTP levels. Unfortunately we have so far been unsuccessful in this attempt

Introduction

In most organisms, including yeast and mammals, an unbalanced or insufficient supply of deoxyribonucleotides triphosphate (dNTP) can cause genomic instability [Reichard 1988]. Consequently, the cellular dNTP pools are subject to stringent regulation. In *Saccharomyces cerevisiae,* DNA lesions that inhibit DNA replication, have been reported to result in a 6 to 8 fold increase in dNTP levels. This increase is responsible for an increased mutation frequency but has also been demonstrated to be essential for cell survival following DNA damage [Chabes *et al.,* 2003; Lis *et al.,* 2008]. In mammals, dNTP can be synthesized by both salvage and *de novo* pathways. Excess dNTPs are degraded by deoxyribonucleotidases where after the resulting deoxyribonucleosides can be excreted from the cell [Reichard 1988; Bianchi and Spychala 2003]. Unlike mammalian cells, yeast cells are not able to synthesize dNTP by the salvage pathway, and no excretion of deoxyribonucleosides has been documented in yeast [Reichard 1988; Chabes and Stillman 2007]. This focuses the regulation of dNTP levels in yeast at the rate-limiting step of the *de novo* pathway; the reduction of ribonucleotides to 2'-deoxyribonucleotides catalyzed by ribonucleotide reductase (RNR) [Larsson and Reichard 1966]. The enzymatic activity of RNR is controlled by several regulatory mechanisms, including allosteric regulation [Kaslan *et al.,* 2002], transcriptional regulation or posttranscriptional inhibitors controlled according to cell cycle or induced by DNA damage. After DNA damage RNR is transcriptionally induced via the Mec1/Rad53 checkpoint pathway by phosphorylation and degradation of the transcriptional repressor of RNR genes, Rfx1. In addition, Mec1 and Rad53 also

inactivate the RNR inhibitor Sml1 via the Dun1 kinase [Zhao *et al.,* 2001; Huang *et al.,* 1998]. The correlation between increased dNTP levels and cell survival following DNA damage has been proposed to be the result of a more efficient DNA translesion synthesis (TLS) following the elevated dNTP concentrations [Chabes *et al.,* 2003]. Progression of the replicative DNA polymerase is stalled by encountering a variety of DNA lesions, increasing the risk of a double strand break [Friedberg and Gerlach 1999]. During TLS, the replicative polymerase is displaced by one or more translesion polymerases allowing bypass of the specific DNA lesions that inhibit replication fork progression. The bypass of DNA damages can be either error-free or error-prone, depending on the nature of the DNA damage as well as the TLS polymerase [Hübscher *et al.,* 2000]. TLS is usually mediated by one polymerase but certain DNA lesions require the action of two different polymerases: One mediating the nucleotide insertion opposite the lesion site and one performing the subsequent extension reaction [Prakash *et al.,* 2005]. In yeast, several polymerases are available for TLS, including polymerase ζ, η and the replicative polymerase δ. The constellation of DNA lesions and polymerases mediating TLS determines whether the process is error-free or error-prone [reviewed in Prakash *et al.,* 2005].

Continued exposure to DNA damaging agents has been demonstrated to result in a continued increase of dNTP levels [Chabes *et al.,* 2003]. Furthermore, increased dNTP levels were demonstrated to be essential for the TLS mediated survival following DNA damage. On this background, we hypothesize that the purpose of the increased levels of dNTP is not only to provide TLS with an abundance of substrate, but also plays a regulatory role in the TLS response. It is conceivable

that the different TLS polymerases have their optimal activity at different dNTP concentrations [Chabes *et al.,* 2003], hence, by increasing the dNTP levels until the DNA lesion has been by-passed, different parts of the TLS is activated or inactivated depending on their optimal dNTP conditions, until one conformation of polymerases is able to by-pass the DNA lesion in either a error-free or error-prone fashion.

The laboratory of our collaborator Dr. Workman (Technical University of Denmark) has recently mapped the transcriptional network controlling the damage response in yeast after treatment with the alkylating agent methyl-methanesulfonate (MMS) [Workman *et al.,* 2006]. Using this transcriptional network we have selected candidate genes that may be responsible for the increase of dNTP levels in response to DNA damage (See table 1; Figure 1). Investigating the role of these genes on the dNTP response and mutagenesis is the first important step in testing our hypothesis.

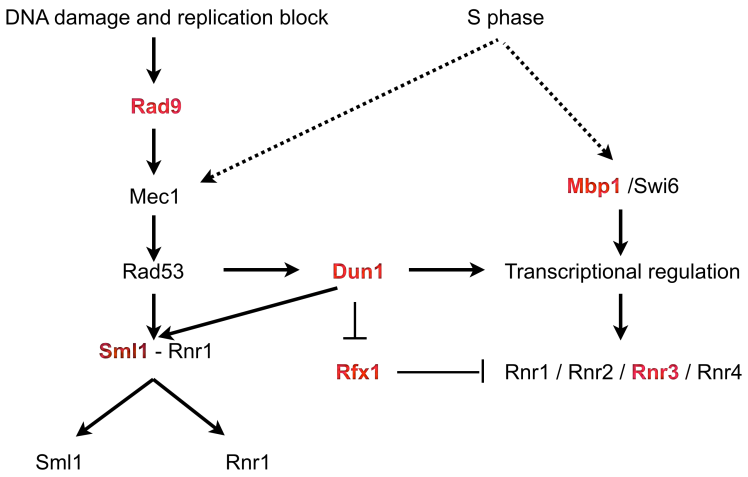

Figure 1. Pathways of interest in the response causing an increase of dNTP levels following DNA damage

Protein	Description
Dun1	Cell-cycle checkpoint serine-threonine kinase required for DNA damage-induced transcription of certain target genes, phosphorylation of Rad55 and Sml1, and transient G2/M arrest after DNA damage; also regulates postreplicative DNA repair
Rad9	Cell cycle checkpoint protein required for cell cycle arrest and DNA damage repair in response to DNA damage. This protein has been found to possess 3' to 5' exonuclease activity, which may contribute to its role in sensing and repairing DNA damage.
Rfx1	Major transcriptional repressor of DNA-damage-regulated genes, recruits repressors Tup1p and Cyc8p to their promoters; involved in DNA damage and replication checkpoint pathway; similar to a family of mammalian DNA binding Rfx1-4 proteins
Mbp1	Transcription factor involved in regulation of cell cycle progression from G1 to S phase, forms a complex with Swi6p that binds to MluI cell cycle box regulatory element in promoters of DNA synthesis genes
Sml1	RNR inhibitor regulated by the checkpoint kinases Mec1/Rad53/Dun1 during DNA damage and S phase. Following phosphorylation Sml1 is degraded
Rnr3	RNR, large subunit; the RNR complex catalyzes the rate-limiting step in dNTP synthesis and is regulated by DNA replication and DNA damage checkpoint pathways via sub-cellular localization of the small subunits

Table 1. Proteins expected to be involved in the dNTP response following DNA damage [Zhao *et al.,* 1998; Zhao *et al.,* 2001; Zhang and Reese 2005; Zaim *et al.,* 2005; Workman *et al.,* 2006].

Materials and methods

Media and strains

Yeast cells were grown in YPD medium (Clontech, Medinova Scientific A/S, Denmark). Cells were plated on YPD agar plates or SD/-Arg + 60 µg/ml canavanine plates (Clontech, Medinova Scientific A/S, Denmark).

S. cerevisiae strains used are listed in table 2 and were obtained from the EUROSCARF collection of deletion strains (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/col_index.html). Genotypes were verified by PCR (data not shown).

	Strain	Genotype
Wild-type	BY4741	MAT**a**, *his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*
DUN1	Y03798	BY4741; *dun1Δ::kanMX4*
RAD9	Y03576	BY4741; *rad9Δ::kanMX4*
RFX1	Y04125	BY4741 *rfx1Δ::kanMX4*
MBP1	Y03753	BY4741; *mbp1Δ::kanMX4*
RNR3	Y01459	BY4741; rnr3Δ::*kanMX4*
REV3	Y02085	BY4741; rev3Δ::*kanMX4*
SML1	Y00512	BY4741; *sml1Δ::kanMX4*

Table 2: *Saccharomyces cerevisiae* strains used in this study

Determination of mutation frequencies

The fluctuation test of the median [von Borstel 1978] was used to determine the mutation frequency to canavanine resistance. Cells grown in suspension was washed in sterile water and approximately 10^8 cells were spread on SD/-Arg + 60 µg/ml canavanine plates and incubated at 30°C for 4 days and the frequency of forward mutation in *CAN1* gene locus was subsequently determined from the number of canavanine resistant colonies counted. Appropriate dilutions of the same cultures were plated on YPD plates to determine the number of viable cells. For each set of experiments five independent cultures of each strain were assayed, and each experiment was repeated at least 3 times. All data are presented as means with standard deviation. These means were calculated by the fluctuation test [von Borstel 1978].

Determination of dNTP levels

Cells grown in suspension were washed twice with sterile water. The number of yeast cells was determined in a Z2 Coulter Counter (Beckham Coulter). 250×10^6 yeast cells were pelleted and resuspended in 1ml 60% methanol solution. The cells were disrupted by 10 consecutive freeze and thaw cycles using liquid nitrogen and warm water and the suspension was incubated at -20°C for 90 minutes, and boiled at 100°C for 3 minutes in order to remove remaining enzyme activity. The lysate was centrifuged at 17000×g for 15 minutes and the supernatant frozen in liquid nitrogen. Methanol was evaporated using a SpeedVac (Thermo Scientific) and the residue was rehydrated in 300 µl Ultra-

pure H_2O (Invitrogen, GIBCO). Determination of cellular dNTP concentration was performed as earlier described [Desler *et al.,* 2007]. Each experiment was repeated at least 3 times

Determination of mutation frequencies and dNTP levels after treatment with MMS

Overnight cultures in stationary phase, were grown in YPD media at 30°C and mutation frequency and dNTP levels were determined as described, for either untreated cells or cells treated with a supplement of 0.025% MMS for 1 hour at 30°C.

For logarithmic phase cells, overnight cultures were diluted 1:50 in YPD for 3 hours at 30°C to ensure that cells were in logarithmic phase. Cells were either assayed directly for mutation frequencies and dNTP content as described above, or following growth in the presence of 0.025% MMS for 1 or 2.5 hours. During growth, cell cultures were diluted in YPD media containing MMS to maintain cells in logarithmic growth by ensuring that OD_{660} did not exceed 0.5.

Statistics

The statistical analysis was performed using Student's unpaired *t*-test. The differences between data sets were considered significant at *p*-values<0.05.

Results

Treatment with MMS causes increased mutation frequency but do not alter the dNTP levels in cells in stationary phase

To determine if DNA lesions cause an alteration of dNTP levels in stationary phase cells, wild type strain, as well as *RAD9*, *MBP1*, *RFX1*, *DUN1* and *RNR3* deletion strains was treated with 0.025% MMS for 1 hour where after the frequency of mutation in the *CAN1* gene was determined and dNTP levels measured. With the exception of the *DUN1* deletion mutant, a significant 2 to 5-fold increase in mutation frequency was demonstrated for the different strains when compared to the frequency of spontaneous mutations in untreated cells (Figure 2). There is no significant difference in mutation frequency between the wild type strain and the different deletion strains in response to MMS treatment.

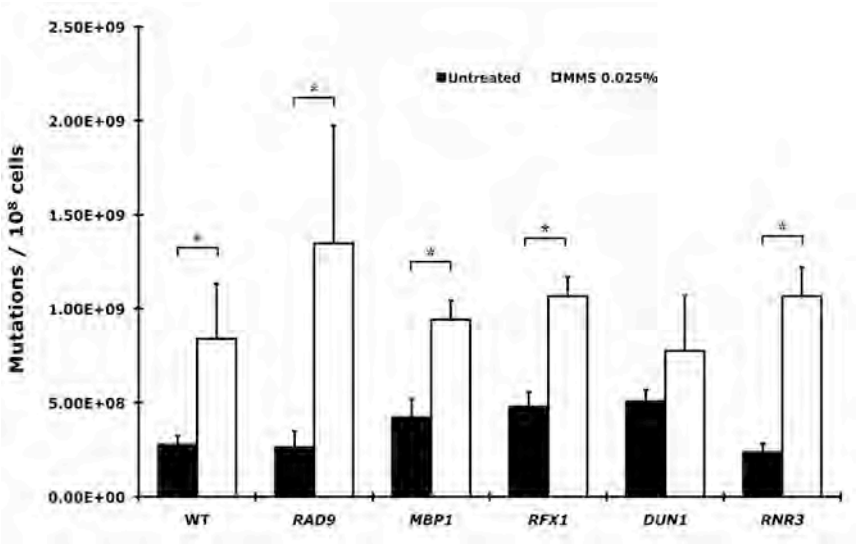

Figure 2. Mutation frequencies in the *CAN1* gene arisen either spontaneously or after 1 hour of treatment with 0.025% MMS. All strains investigated were in stationary phase and displayed a 2 to 5 fold increase in mutation frequency after MMS treatment (n=3 error-bars indicate S.D. (*) Denotes significant difference. The statistical analysis was performed using Student t-test. A difference at $p < 0.05$ was considered significant.)

For the *RAD9* deletion strain, there is no significant difference in the dNTP levels between cells in stationary phase and corresponding cells treated with MMS . For the wild type strain and *MBP1*, *RFX1*, *DUN1* and *RNR3* deletion strains, treatment with MMS for 1 hour leads to a 1.5 to 2 fold increase of one or more of the dNTP pools (Figure 3). The size of the dGTP and dCTP pools were most affected by the MMS treatment where all investigated strains, with the exception of the *RAD9* deletion strain, displayed significant increased dGTP levels and all investigated strains, with the exception of wild type and the *RAD9* deletion strain displayed significant increased dCTP levels in response to MMS treatment. For the dTTP pool, only wild type, the *RFX1* and the *RNR3* deletion strains displayed a significant increase in response to MMS treatment. For the dATP pool, no increase was measured. On the contrary, the *RNR3* deletion strain displayed a significant decrease in dATP levels as a result of the MMS treatment.

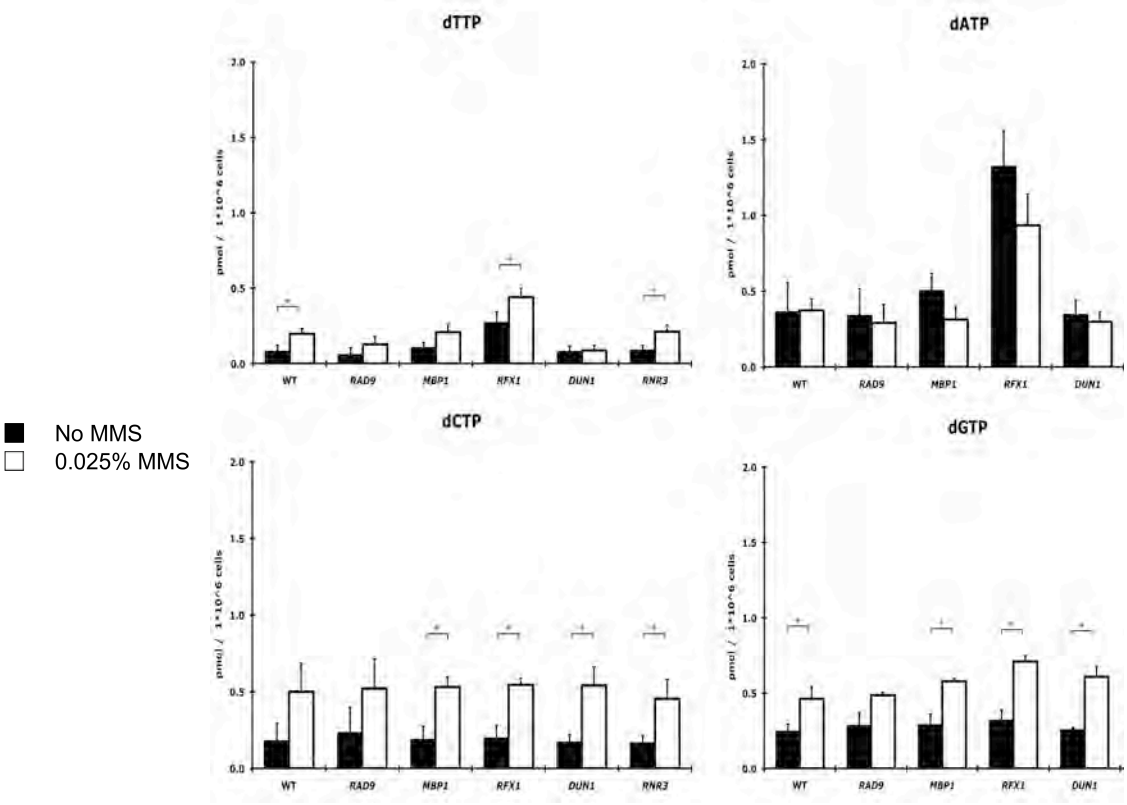

Figure 3. Nucleotide pools in wild type and knock out strains in stationary phase, treated for 1 hour with or without 0.025% MMS. From top left to bottom right are shown the dTTP, dATP, dCTP and dGTP levels, respectively. (n=3 error-bars indicate S.D. (*) Denotes significant difference. The statistical analysis was performed using Student t-test. A difference at $p < 0.05$ was considered significant.)

Treatment with MMS for 1 hour causes increased dNTP levels but do not increase mutation frequency in cells in logarithmic phase

To investigate possible link between treatment with DNA damaging agents and dNTP pools in logarithmic phase cells, wild type strain as well as *RAD9, MBP1, RFX1, DUN1* and *RNR3* deletion strains were treated with 0.025% MMS for 1 hour during logarithmic growth and assayed for dNTP content (Figure 4). With exceptions, all investigated strains displayed a significant increase in dTTP, dCTP and dGTP levels as a result of the MMS treatment. The affected pools were

increased by a magnitude of 5 to 30 fold. The exceptions include the dTTP pool of *RFX1* and *DUN1* deletion strains and the dGTP pool of the *RNR3* deletion strain where no significant difference was measured between untreated and cells treated with MMS. As was the case for stationary phase cells treated with MMS, the dATP pool of logarithmic phase cells was not as affected as the dTTP, dCTP and dGTP pools by the MMS treatment and only the *RAD9* and *DUN1* deletion strain displayed a significant increase of dATP levels of a magnitude of 10-14 fold in response to MMS treatment.

When comparing the dNTP levels of logarithmic phase wild type cells with the investigated deletion strains, the response to MMS treatment is only significantly different from the wild type strain in *RAD9* and *DUN1* deletion strain. The dTTP pool of the *RAD9* deletion strain is 1.6 fold lower than in the wild type strain and the dGTP pool of the *DUN1* deletion strain is 2.7 fold lower than in the wild type strain. These results indicate that none of the selected genes from the global gene expression study (Table 1) was directly responsible for the MMS induced dNTP response.

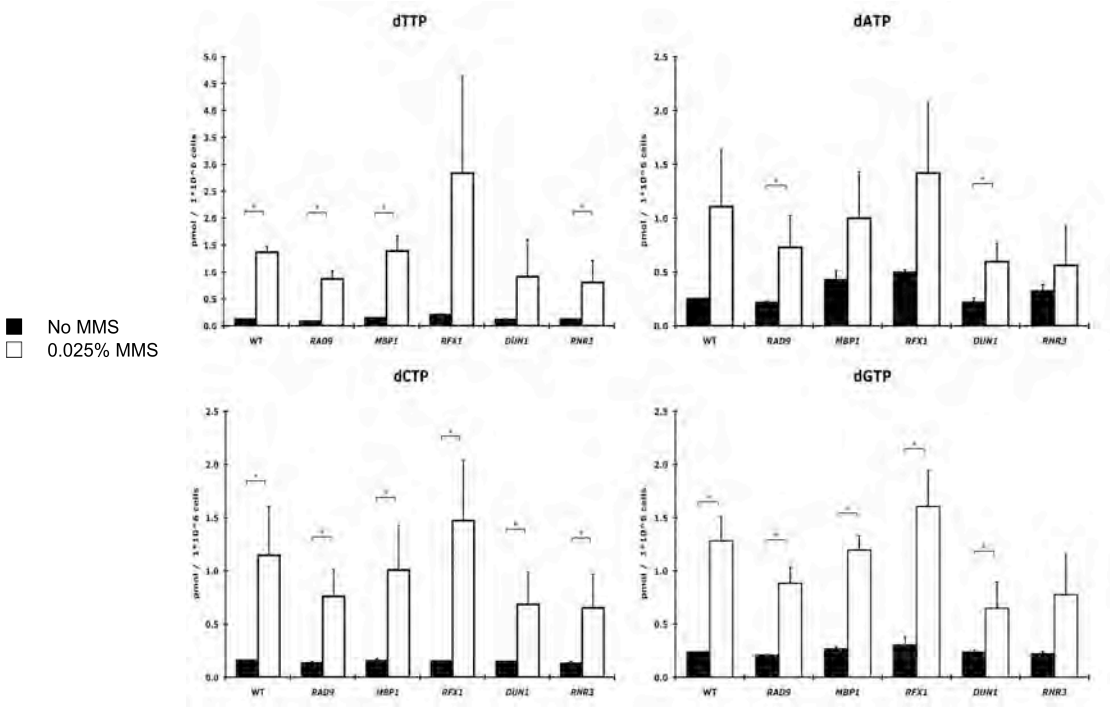

Figure 4. Nucleotide pools in wild type and knock out yeast strains in logarithmic phase treated for 1 hour with or without 0.025% MMS. From top left to bottom right are shown the dTTP, dATP, dCTP and dGTP levels, respectively. A 5-30 fold increase of the dNTP levels of MMS treated strains was demonstrated in response to MMS treatment. With exception of the dTTP pool of *RAD9* and the dGTP pool of the *DUN1* deletion strain, no significant difference exist between treated wild type strain and deletion strains (n=3 error-bars indicate S.D. (*) Denotes significant difference. The statistical analysis was performed using Student t-test. A difference at $p < 0.05$ was considered significant.)

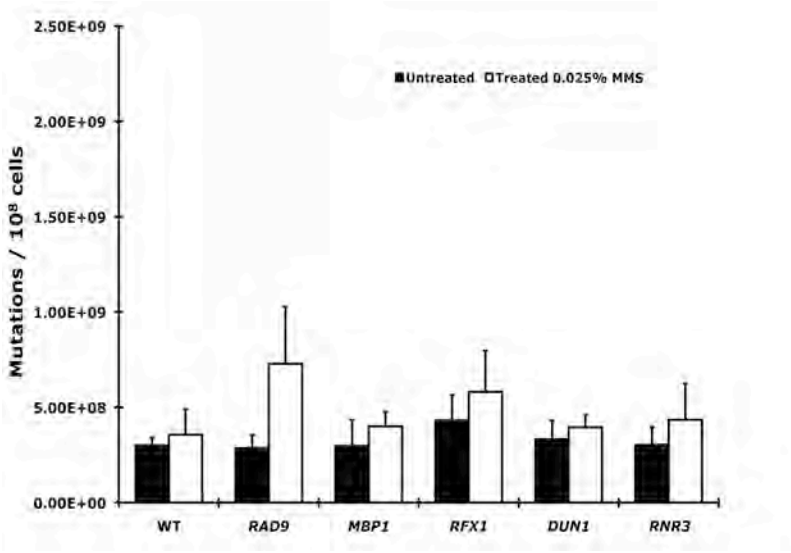

Figure 5. Mutation frequencies either occurring spontaneously or after 1 hour of treatment with 0.025% MMS. The results represent the average of three sets of independent experiments with standard deviation. All strains investigated were in logarithmic phase. No significant difference was measured between treated and untreated cells (The statistical analysis was performed using Student t-test. A difference at $p < 0.05$ was considered significant.)

Treatment of wild type as well as *RAD9, MBP1, RFX1, DUN1* and *RNR3* deletion strains with 0.025% MMS for a 1 hour period, was demonstrated to be correlated with a 10-14 fold increase in dNTP levels (Figure 4), but no significant increase in mutation frequencies was detected as a result of the MMS treatment (Figure 5). This is in contrast to same strains in stationary phase, where MMS treatment only increased the dNTP levels 1.5 to 2 fold while the mutation frequency as increased 2 to 5 fold (Figure 2-3).

dNTP level continues to increase during 2.5 hour of MMS treatment

When treating logarithmic growing wild type as well as *RFX1, SML1, REV3* deletion strains with 0.025% MMS for a period of 2.5 hours, a continued increase of dNTP levels was demonstrated (Figure 6).

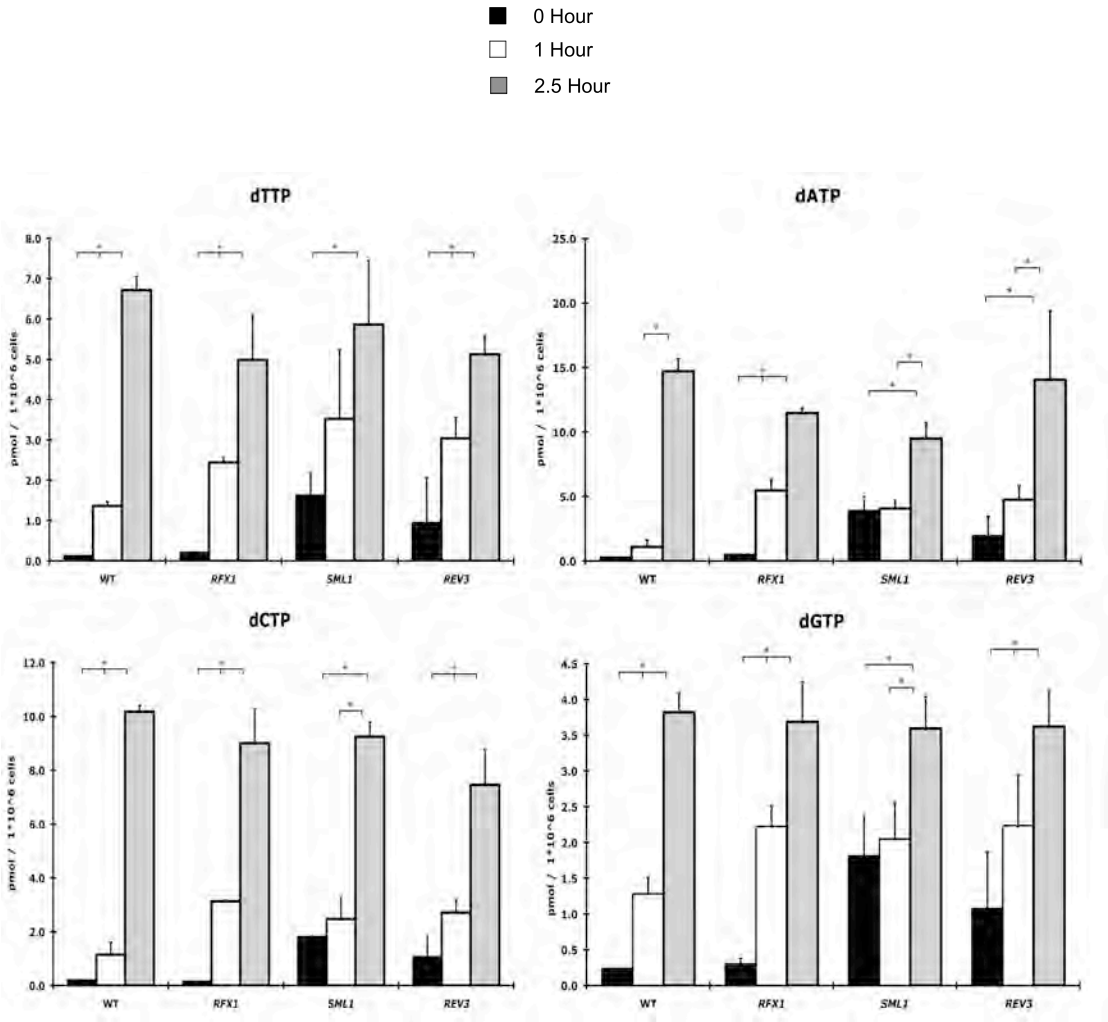

Figure 6. Nucleotide pools in wild type and deletion strains in logarithmic phase, untreated or treated for 1 or 2.5 hours with 0.025% MMS. From top left to bottom right are shown the dTTP, dATP, dCTP and dGTP levels, respectively. A time dependent increase of dNTP levels was demonstrated for all strains (n=3 error-bars indicate S.D. (*) Denotes significant difference. The statistical analysis was performed using Student t-test. A difference at $p < 0.05$ was considered significant.).

During the course of the 2.5 hour of MMS treatment, especially the wild type strain displayed a dramatic increase of dNTP levels, where treated cells contained 20-60 fold higher concentrations of the four dNTP when compared to untreated cells.

For the *RFX1* and *SML1* deletion strains, the concentrations of dNTP after 2.5 hours of MMS treatment is comparable to the dNTP concentration for wild type cells treated correspondingly. The same is not true for the dNTP levels of untreated cells where the dNTP levels of untreated *SML1* deletion strains are 9-16 fold higher. After 1 hour of MMS treatment the dNTP levels of untreated *SML1* and *RFX1* deletion strains are 2 to 5 fold higher than the dNTP concentration of corresponding wild type cells.

Interestingly, MMS treatment not only increases dNTP levels but also alters the ratio between the four deoxyribonucleotide triphosphate pools (Table 3)

	dTTP	**dATP**	**dCTP**	**dGTP**
0 hour	1	2.4	1.6	1.9
1 hour	1	0.8	0.8	0.9
2.5 hours	1	0.9	0.9	1.0

Table 3. The ratio of dTTP/dATP/dCTP/dGTP pools of wild type strains in logarithmic phase, treated with 0.025% MMS for a period of 0, 1 and 2.5 hours. Ratios are normalized to dTTP content

Mutation frequencies during a 2.5-hour treatment with MMS

The mutation frequencies of logarithmic growing wild type, *RFX1*, *SML1* and *REV3* deletion strains was determined for untreated cells and cells treated with

MMS for 2.5 hours (Figure 7). Wild type, *RFX1* and *SML1* deletion strains was measured to have a 5 to 6 fold increase in mutation frequencies when comparing to untreated cells. This is in contrast to investigated strains treated with MMS for 1 hour, where no significant difference were measured when compared to untreated cells. This indicated that the capabilities of these cells to repair MMS induced lesions had been saturated after 2.5 hours of treatment. In contrast, only a 3 fold increase of mutation frequencies were measured between untreated *REV3* deletion strains and *REV3* deletion strains treated with 0.025% MMS for 2.5 hours. In comparison, the mutation frequency of the *REV3* deletion strain treated with MMS was 5-6 fold lower than corresponding mutation frequencies of wild type, *RFX1* or, *SML1* deletion strains.

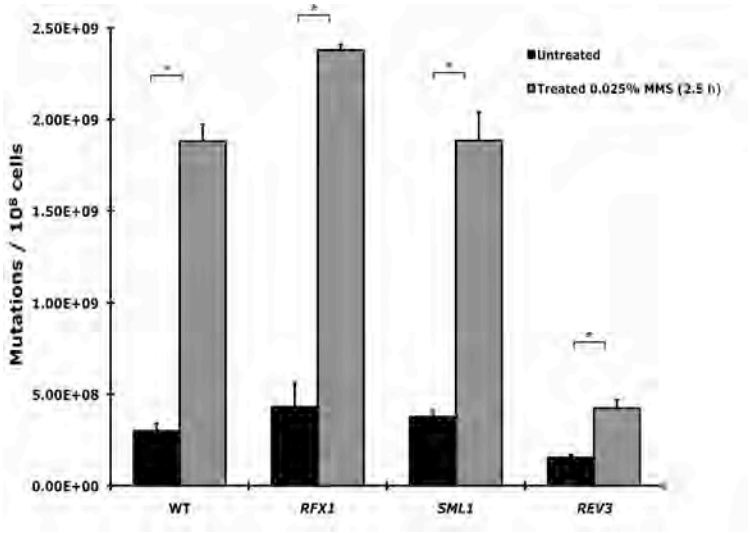

Figure 7. Frequencies of mutations either occurring spontaneously or after 2.5 hours of treatment with 0.025% MMS. (n=3 error-bars indicate S.D. (*) Denotes significant difference. The statistical analysis was performed using Student t-test. A difference at $p < 0.05$ was considered significant.).

Discussion

Treatment with MMS for 1 hour, induces dNTP levels of cells in logarithmic phase, but not cells in stationary phase

Treatment of logarithmic phase cells with the alkylating agent MMS induces a 5-30 fold induction of dNTP levels depending on the genotype and pool measured. In stationary cells, same treatment only induces an up to 2 fold increase of the dNTP pool. This indicates that treatment with MMS is followed by an increase of dNTP concentrations, but primarily in dividing cells. We hypothesize that the dNTP response is initiated by a stalling of the replication fork. For a cell culture with cells in stationary phase only a fraction of cells are replicating, explaining why a dNTP response is not seen in these cells. To test this hypothesis it would be necessary to use synchronized cells that were released and treated with MMS at different points of the cell cycle. This would answer whether a replication fork must be present to initiate an increase of the dNTP concentration following MMS treatment.

The levels of dNTP continues to increase during a 2.5 hour treatment with MMS

By treating logarithmic phase cells with MMS over a period of 2.5 hours, a time dependent increase of dNTP was demonstrated for all strains investigated. In wild type strain, the dNTP content increased 20-60 fold during 2.5 hours of treatment. Interestingly, MMS treatment does not only induce an increase in dNTP concentration, but also alters the ratio between the four deoxyribonucleotides. Both an increase of dNTP concentration and a change of

ratio of dTTP:dATP:dCTP:dGTP can lead to mutagenic events [Reichard 1988]. It is therefore possible that the increased and imbalanced pools of dNTP can result in mutations by its own. To verify this finding it is important to perform additional measurements in order to have sufficient material to make a more precise statistical analysis of the ratio differences.

The differences in dNTP levels reported in this paper are much higher than reported in other works [Chabes *et al.,* 2003; Lis *et al.,* 2008]. These differences are most likely due to the fact that two different approaches have been utilized in the determination of dNTP levels. In this paper we have utilized the Klenow mediated dNTP determination, whereas other groups have utilized HPLC methods. The difference between strains reported in this paper must therefore also be verified by HPLC determination of dNTP levels.

RFX1 and *SML1* deletion strains do not express the two RNR inhibitors Rfx1 and Sml1 respectively. As a result the dNTP levels of untreated *SML1* deletion strains are 9-16 fold higher than corresponding dNTP levels of wild type strains. In contrast the dNTP levels of *RFX1* deletion strains are only 2 fold higher. Even though untreated *RFX1* and *SML1* deletion strains have an increased dNTP concentration compared to the wild type strain, the two RNR inhibitor knock-out strains still responds to MMS mediated DNA damage, indicating that the dNTP response is not mediated by a down-regulation of either of these RNR inhibitors.

The mutational response to 2.5 hours of MMS treatment can be explained by the increase of dNTP levels

In spite of treatment with MMS for 1 hour and a resulting increase in the concentration of dNTP levels, logarithmic phase cells do not present a significant increase in mutation frequency when compared to untreated cells. It is evident that these strains are able to tolerate the DNA lesions induced by MMS and altered levels of dNTP. An explanation would be that the increased dNTP levels facilitates DNA repair or damage by-pass in an error-free fashion, but to prove that the increase in dNTP concentration is necessary for this tolerance, we would have required a strain which did not induce the dNTP levels in response to MMS treatment. By using this strain it would be possible to investigate if 1 hour of MMS treatment would be tolerated equally well as in strains that were able to induce dNTP levels following treatment with MMS. Even though we investigated several candidates we have yet to find such a strain.

After 2.5 hours of MMS treatment all investigated strains, with the exception of the *REV3* deletion strain, displayed an increase in mutation frequencies. According to our hypothesis, different TLS polymerases have their highest activity at different dNTP concentrations and the dNTP levels will regulate the TLS response. If the signal for increased dNTP levels is mediated by a stalled replication fork, the dNTP levels would continue to increase until a suiting TLS response was found for bypass of the DNA lesion whereby the replication fork could be continue its progression. In the progression of TLS response to MMS damage, a moderate increase in dNTP levels after 1 hour of MMS treatment appears to have activated error-free TLS, while the further increased dNTP levels

after 2.5 hours of MMS treatment utilizes error-prone TLS in response to the treatment (Figure 8).

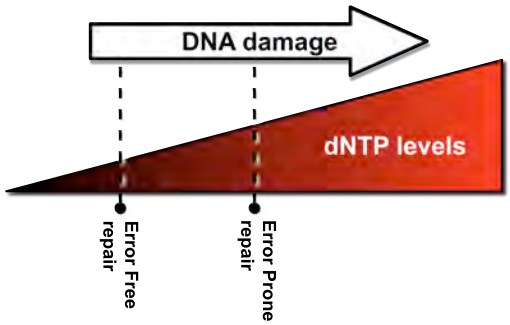

Figure 8. Model of the hypothesized relationship between dNTP levels, following DNA damage inducing agents, and the TLS response. According to the model, a moderate increase of dNTP concentrations facilitate error-free repair or TLS, while a further increase of dNTP concentrations provide better conditions for the error prone repair or TLS

When treated with MMS for 2.5 hours the *REV3* deletion strain only presented a 3-fold increase in mutation frequency. This indicates either that the *REV3* deletion strain had not saturated its ability to perform error-free repair or DNA lesion bypass after 2.5 hours of MMS treatment or that the cells where unable to initiate error-prone repair or DNA lesion bypass once the error-free pathways was saturated, leading to a decreased survival rate of the culture. *REV3* encodes the catalytic subunit of the error-prone TLS polymerase ζ [Sakamoto *et al.,* 2007], and it is therefore highly probable that the mutation response seen in the other strains was mediated by this part of the TLS pathway.

If we can substantiate our hypothesis and demonstrate that dNTP levels are instrumental when deciding which TLS response to be utilized, this will open a new understanding of the role of the dNTP levels in mutagenesis.

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Paper IV

Desler, C., Suravajhala, P., Sanderhoff, M., Rasmussen, M. and Rasmussen, L.J. (2009) *In Silico* screening for functional candidates amongst hypothetical proteins. *BMC Bioinformatics,* Revision submitted.

In Silico screening for functional candidates amongst hypothetical proteins.

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Abstract

Background

The definition of a hypothetical protein is a protein that is predicted to be expressed from an open reading frame, but for which there is no experimental evidence of translation. Hypothetical proteins constitute a substantial fraction of proteomes of human as well as other eukaryotes. With the general belief that the majority of hypothetical proteins are the product of pseudogenes, it is essential to have a tool with the ability of pinpointing the minority of hypothetical proteins with a high probability of being expressed.

Results

Here, we present an *in silico* selection strategy where eukaryotic hypothetical proteins are sorted according to two criteria that can be reliably identified *in silico*: the presence of subcellular targeting signals and presence of characterized protein domains. To validate the selection strategy we applied it on a database of human hypothetical proteins dating to 2006 and compared the proteins predicted to be expressed by our selecting strategy, with their status in 2008. For the comparison we focused on mitochondrial proteins, since considerable amounts of research have focused on this field in between 2006 and 2008. Therefore, many proteins, defined as hypothetical in 2006, have therefore later been characterized as mitochondrial.

Conclusions

Among the total amount of proteins hypothetical in 2006, 21% have later been experimentally characterized and 6% of those have been shown to have a role in a mitochondrial context. In contrast, among the chosen hypothetical proteins from the 2006 dataset, predicted by our strategy to have a mitochondrial role, 53-62% have later been experimentally characterized, and 85% of these have actually been assigned

a role in mitochondria by 2008.

Therefore our *in silico* selection strategy can be used to select the most promising candidates for subsequent *in vitro* and *in vivo* analyses.

Background

According to the Human Genome Organization (HUGO), the human genome is predicted to consist of 19599 protein-encoding genes [1, Human Genome Project (http://www.hugo-international.org/)]. A substantial part of these genes is predicted to encode a group of proteins, where translation has not been demonstrated and the proteins themselves have not been characterized. This group of proteins is accordingly defined as hypothetical. Although many of the listed hypothetical proteins most likely are predicted products of pseudogenes, there is a reasonable probability that a number of the listed hypothetical proteins are truly novel and can perform uncharacterized biological functions. Consequently, the putative importance of hypothetical proteins is not negligible.

Several *in silico* methods are available for descriptive predictions of proteins with unknown function. These include studies of homology, database searches for orthologs, or the presence of characterized functional domains or motifs within the protein [2]. Most often false positives will occur and predictions must be substantiated by *in vitro* and/or *in vivo* experiments to validate and further characterize predicted functionality. The *in silico* methods are designed for functional prediction of a protein, but not specifically designed to ascertain whether a protein is hypothetical or not. When screening hypothetical proteins for novel translatable candidates *in silico* methods are therefore rarely used and the researcher often performs the screen with laborious *in vitro* and/or *in vivo* experiments.

In the present study, we propose an *in silico* screening strategy for eukaryotic systems, in which novel translatable candidates can be selected from a group of hypothetical proteins. The strategy is based on *in silico* methods normally used to make functional predictions of proteins, which include search for presence of sub-cellular targeting signals and for presence of characterized protein domains. Especially targeting signals and, to a lesser extent, protein domains can be predicted with high probability. The occurrence of either targeting signals or identifiable protein domains can also be present in pseudogenes as a result of gene duplication. However, we hypothesize that the risk of a hypothetical protein being a pseudogene is greatly reduced when both targeting signals and protein domains are identified in the transcript, especially if the protein domain architecture suggests a relevant function in the predicted sub-cellular compartment. Selection of hypothetical proteins based on a combination of both these factors should therefore greatly increase the success rate of discovering true functional proteins with roles in subcellular compartments among hypothetical proteins. Due to the design of the selection strategy it is ineffective for identifying proteins without localization signals, and this must be taken into consideration.

To exemplify our selection strategy we have chosen mitochondria as the targeted sub-cellular compartment. Within recent years, a substantial amount of work has been invested in compiling a near complete list of mitochondrial proteins in humans. This has resulted in the establishment of the MitoCarta database (http://www.broad.mit.edu/pubs/MitoCarta/) [3]. The total number of genes encoding mitochondrial proteins is according to MitoCarta at least 1013 [3]. Mitochondria are semiautonomous organelles present in almost all eukaryotic cells ranging from a

single copy to several thousands. Mitochondria contain their own autonomous genome, which encodes 37 of these proteins. The reminder is encoded by nuclear DNA and imported into mitochondria. Examples of mitochondrial functions include ATP production by oxidative phosphorylation, β-oxidation of fatty acids, metabolism of amino acids and of lipids. Furthermore, mitochondria have a prominent role in apoptosis.

With the exception of proteins encoded by the mitochondrial genome, proteins are translated in the cytosol from their corresponding mRNA. Many proteins are transported to specific parts of the cell where they function in context of the sub-cellular compartment. The sub-cellular localization of proteins can be facilitated by specific targeting peptides. There are two types of targeting peptides, the presequences and internal targeting signals. Presequences are often localized at the N-terminal whereas internal targeting signals can be distributed throughout the protein sequence [4-6]. The mitochondrial membrane contains translocases for recognition and import of nuclear-encoded mitochondrial proteins. The translocase of the outer mitochondrial membrane (TOM complex) is responsible for recognition and initial import of nuclear-encoded mitochondrial proteins (reviewed in [7]). Mitochondrial precursor proteins posses either an N-terminal presequence or internal targeting signals. Both types of targeting peptides, N-terminal or internal, are recognized by different import receptors of the TOM complex. N-terminal presequences generally have a length of 6-85 amino acid residues, enriched in Arg, Ser and Ala, while negatively charged amino acids are rarely present [8]. N-terminal presequences form positively charged amphiphilic α-helices when bound to import receptors on the mitochondrial surface [9], and upon mitochondrial import, presequences are removed

by proteolysis (reviewed in [10,11]). Even though binding of different parts of the TOM complex to varying internal targeting signals has been shown [12], a common motif for an internal targeting signal has still to be elucidated,

In order to validate that our *in silico* selection strategy can predict functional candidates among hypothetical proteins we chose to focus on proteins with a predicted mitochondrial function. We have utilized an existing database of hypothetical proteins assembled in 2006 [13]. From this database we selected all hypothetical proteins predicted to be localized in human mitochondria due to the presence of a suspected mitochondrial N-terminal presequence. These selected proteins were then investigated for the presence of potentially functional protein domains. We predict that the sub-group of hypothetical proteins, with both a mitochondrial N-terminal presequence and potentially functional protein domains has a high probability of being expressed and having a function in a mitochondrial context.

All proteins investigated were hypothetical in 2006. However, between 2006 and 2008, many proteins have been experimentally characterized or removed from the database as they have been predicted to be products of pseudogenes. This increases the probability that the 2006 dataset of hypothetical proteins includes a large number of proteins that are now (as of 2008) classified as mitochondrial. By applying the selection strategy on the 2006 dataset, we are able to compare the resulting predictions with the factual *in vitro* and/or *in vivo* characterizations of the proteins performed from 2006 to 2008. Effectiveness of selection strategy can be demonstrated by comparing proteins selected from the 2006 dataset with the number of these

proteins that, as of November 2008, are demonstrated to be translated, mitochondrial or proven to be a pseudogene.

Methods

We have utilized a database of proteins extracted from GenBank in August 2006. At the time of extraction, all proteins were defined as hypothetical and all sequences were crosschecked and annotated [13]. In November 2008, the status of each individual protein was reinvestigated and entries of the 2006 database that later have been identified as duplicates were removed. The entries of the 2006 dataset were divided into three groups according to their individual status in November 2008: Hypothetical proteins, characterized proteins and proteins discovered to be pseudogenes and therefore are removed by GenBank. These three groups are in the following collectively referred to as the 2008 dataset.

Several prediction programs have been designed to predict the localization of eukaryotic proteins. In table 2, we have listed a selection of available programs, which have been reported to have a medium to high prediction accuracy. To exemplify the occurrence of hypothetical proteins with functional targeting peptides, hypothetical proteins from the 2006 dataset were analyzed using pTarget. The pTarget program [http://bioapps.rit.albany.edu/pTARGET/] predicts protein targeting to nine different sub-cellular locations including mitochondria. Prediction is based on the occurrence of specific Pfam domains earlier determined to be location specific. pTarget, can predict 68-87% of the true positives at accuracy rates of 96-99% [19,20]. Since the program makes its predictions based on putative location-specific Pfam domains, and not necessarily targeting peptides verified in experimental setups, we have chosen to use the program only to illustrate how hypothetical proteins can be sorted by predicted localization.

In contrast to pTarget, the TargetP program [http://www.cbs.dtu.dk/services/TargetP/] predicts mitochondrial localization using the N-terminal sequence information only, with a success rate of predictions of 90% [21]. TargetP was used to screen the 2006 dataset for functional mitochondrial targeting peptides.

All hypothetical proteins predicted to have a mitochondrial targeting peptide by TargetP, were further characterized using the SMART program [http://smart.embl-heidelberg.de/]. The SMART program identifies protein domains from a database of manually annotated known protein domains, locating proteins with identical domain architecture [22, 23].

We hypothesize that hypothetical proteins, predicted to contain both a mitochondrial N-terminal presequence and functional protein domains have a high probability of being functional in a mitochondrial context. To verify our hypothesis, we used the 2006 dataset of the, then, hypothetical proteins. Using TargetP, we selected proteins having a high probability of containing a mitochondrial N-terminal presequence. For the resulting subset of proteins we used SMART to search for the presence of functional protein domains. Comparing with protein status according to the 2008 dataset, we determined the percentage of selected proteins that had either been removed or experimentally characterized after 2006. Furthermore, if proteins had been experimentally characterized, we determined if they had been found to be functional in a mitochondrial context.

Results and discussion

After removing proteins found or predicted to be duplicates of already existing proteins, the 2006 dataset of hypothetical proteins contains 5860 proteins. According to GenBanks current annotation (November 2008) of the same group of proteins, 1455 of the 5860 proteins annotated as hypothetical in 2006, are still hypothetical, while 1215 proteins have been experimentally characterized and 3190 proteins have been removed by GenBank as they have been identified as pseudogenes (See Additional file 1).

pTarget was used to predict the distribution of human hypothetical proteins from the 2006 dataset and 2008 dataset (Table 1). A third of the hypothetical proteins from both datasets were predicted to be nuclear, while only 2-3% were predicted to be localized to the endoplasmic reticulum, and 5% of investigated hypothetical proteins were predicted mitochondrial. We only use pTarget to exemplify how human hypothetical proteins can be sorted based on their predicted cellular localization. This is important since our selection strategy is limited to proteins targeted for a sub-cellular localization. Using pTarget as an indicator only, we are able to demonstrate that proteins predicted to be localized to lysosomes, golgi, peroxysomes, mitochondria or endoplasmic reticulum, consists of 32% of the 2006 dataset. When including proteins predicted to be secreted, proteins targeted for the plasma membrane or nucleus, this includes 87% of the 2006 dataset. This indicates that the selection strategy, according to pTarget, can be applied on up to 87% of the dataset.
pTarget is not an optimal tool for investigating hypothetical proteins. pTarget screens for putative Pfam domains that are related to a specific cellular localization but not necessarily for complete targeting signals. If a hypothetical protein turns out to be a

pseudogene, it will most likely consist of gene duplications. Even though several Pfam domains related to specific cellular locations are found in a protein, the complete targeting signal is not guaranteed to be complete and functional.

TargetP predicts probability of mitochondrial localization based solely on mitochondrial specific presequences. These motifs do not necessarily require *cis* or *trans* acting domains in order to be fully functional mitochondrial targeting signals. Accordingly, if a hypothetical protein is predicted to be localized to the mitochondria, there is a reasonable probability that a corresponding expressed protein would be localized to this organelle even though it may still be the product of a pseudogene. Screening the 2006 dataset of human hypothetical proteins with TargetP we found a total of 1139 proteins predicted to be localized to mitochondria (See Additional file 1). TargetP places all of the predicted proteins into reliability classes, ranging from 1 to 5, where 1 indicates the strongest prediction [21]. We have focused on the total of 538 proteins belonging to reliability class 1 (52 proteins), 2 (204 proteins) and 3 (282 proteins) (See Additional file 1).

From reliability class I to III, 315 of 538 proteins (59%) selected by TargetP, had been removed, while 75 of 538 proteins (14%) had been characterized and 32 of 538 proteins (6%) had been characterized as mitochondrial. When focusing on proteins predicted by TargetP to be in reliability class I, 15 of 52 proteins (29%) had been removed, 18 of 52 proteins (35%) had been characterized and 14 of 52 proteins (27%) had been characterized as mitochondrial (See Additional file 1). From the 2006 dataset of hypothetical proteins, 67 are listed in MitoCarta as characterized mitochondrial proteins (See Additional file 1). TargetP was successful in identifying

32 of the total of 67 mitochondrial proteins, but did also select 315 proteins that after 2006 have been removed. When focusing on proteins belonging to reliability class I, 14 of 67 mitochondrial proteins were identified, while 15 proteins had been removed. This demonstrates that TargetP is efficient in finding mitochondrial proteins, but it is not suitable for screening hypothetical proteins for novel translatable candidates. To be able to annotate the selected hypothetical proteins we screened proteins from reliability class I to III with the SMART program to determine the presence of known protein domains. We ignored proteins only containing transmembrane domains, coiled coil regions, signal peptides and/or segments of low compositional complexity, as these regions are not unique protein domains.

Three groups of proteins where constructed from the 538 proteins investigated. Group I consists of 20 proteins, predicted by TargetP to belong to reliability class 1 and to contain identifiable protein domains according to SMART prediction. Group II consists of 56 proteins, predicted by TargetP to belong to reliability class 1 and 2 and to contain identifiable protein domains. Group III contains 100 proteins that are predicted by TargetP to belong to reliability class 1, 2 and 3 and to contain identifiable protein domains. Group III therefore contains all proteins of group I + II and group II contains all proteins of group I. The construction of these three groups allows us to comment on how reliable the prediction of cellular localization should be in order to get a good result from our selection strategy.

The three groups of selected proteins were, together with the 5860 hypothetical proteins from the 2006 dataset, compared with their corresponding 2008 annotations. The comparison include localization of experimentally characterized proteins and

proteins removed due to being the predicted result of pseudogenes or similarity to an existing protein (Table 3).

25% of the 5860 proteins were in November 2008 still annotated as hypothetical, 21% had been experimentally characterized and 54% were removed. Of the experimentally characterized proteins, 67 proteins or 6% were listed in MitoCarta as mitochondrial. Group III, contains 100 proteins, where 36% in November 2008, were still hypothetical, 53% had been characterized and 11% had been removed. Of the characterized proteins 45% or 24 proteins were listed in MitoCarta as mitochondrial. The values obtained clearly demonstrate, that our strategy using a selection based both on the presence of a mitochondrial presequence and identifiable protein domains is very efficient for extracting hypothetical proteins with a functional role in mitochondria. Applying the selection strategy on the 2006 dataset identified 24 of 67 proteins that later have been categorized as mitochondrial. Furthermore the percentage of removed proteins is 5-fold lower for group III when compared with the 2006 dataset and the percentage of characterized proteins is concurrently 2.5 fold higher. When investigating group II and group I proteins, selected by our strategy it is evident that the percentage of removed proteins is diminished to 9% and 5% respectively, and the percentage of characterized proteins is increased to 65% and 64% respectively. The prevalence of mitochondrial proteins within the number of characterized proteins increases from 6% for the total 2006 dataset to 45%, 58% and 85% for group III, group II and group I respectively.

When increasing the selectivity of the applied prediction models, in our case by focusing on group II and especially group I proteins, it is evident that the probability

of finding hypothetical proteins, which will have a function in the predicted sub-cellular compartment is increased. However, it is also evident that the higher the selectivity, the lower the total number of identified proteins with the desired functionality. For group III proteins, 24 out of a total of 67 mitochondrial proteins were discovered. For group I, only 11 proteins out of the 67 mitochondrial proteins were discovered.

The three groups of investigated proteins contain a total of 36 proteins that in November 2008 was still annotated as hypothetical. According to our selecting strategy, these proteins are predicted to be expressed and to have roles in a mitochondrial context. To investigate the potential of these proteins, we investigated the protein domains of 6 hypothetical proteins of group I to see if they would suggest a mitochondrial function for the relevant protein (Table 4). 4 out of the 6 proteins each contain one domain that are experimentally characterized in mitochondria and therefore may have putative mitochondrial functions. The four domains were found to be a Complex I- Lyr domain, a Methyltransferase 12 domain, a Sel 1 domain and a DUF1640 domain. The Complex I-LYR domain is present in a family of proteins including the mitochondrial NADH-ubiquinone oxidoreductase complex I. Methyltransferase 12 domain is found in a variety of methyltransferases including one functioning in mitochondria. Sel I like repeats domain is found in a vast amount of proteins including HSP70, HSP90, and in mitochondrial Tom 70 import receptor. DUF1640 domain is present in the mitochondrial protein FMP32 found in *Saccharomyces cerevisiae*. [24, 25, 27, 30, 31]

Conclusion

A hypothetical protein may have a yet, uncharacterized role in a biological context or simply be the predicted result of a pseudogene and with no biological relevance. In order to screen a dataset of hypothetical proteins, we propose a simple selection strategy where proteins are selected on the basis of well-characterized targeting peptides and protein domains. We have utilized a database of hypothetical proteins dating from 2006 and reviewed their annotated status in 2008. Accordingly, we can verify our selection strategy by reviewing the proteins that were hypothetical in 2006, but have been experimentally characterized by November 2008. We chose to screen for hypothetical proteins predicted to be mitochondrial since considerable amounts of work have been performed within the last couple of years to build extensive databases of the human mitochondrial proteome, summarized in works like MitoCarta. From the 2006 dataset, 5860 hypothetical proteins were identified, and from this dataset, we identified 100 proteins that we believe, have a high probability of being expressed mitochondrial proteins, based on our selection strategy. This list is not exhaustive as, for instance, hypothetical proteins with mitochondrial internal targeting signals but no mitochondrial presequence, were not considered. When compared to the 2008 dataset, we found that 53 of the 100 hypothetical proteins predicted to be mitochondrial have now been characterized, and 45% of those were found to be mitochondrial. In comparison, only 6% of the characterized proteins from the 2006 dataset have been categorized as mitochondrial in the same time period. Increasing the selectivity of TargetP increases the incidence of characterized mitochondrial proteins to 85%, but unfortunately decreases the total number of mitochondrial proteins identified.

Investigating identified protein domains of 6 of the 36 hypothetical proteins predicted to be mitochondrial, we found a subset of 4 proteins having a strong mitochondrial signature in their identified protein domains. It is our opinion that these proteins are very interesting candidates for further experimental characterizations.

In present work we have applied our selection strategy in the search for human mitochondrial proteins. From the characterized proteins we were able to verify the fidelity of our *in silico* selection strategy. The strategy is general and can be used to identify hypothetical proteins with a high probability of having a role in any organelle compartment where the internal targeting signals are characterized. Furthermore, many of the targeting signals and identifiable protein domains that are valid in human cells, are also valid in other eukaryotes. Our selection strategy can therefore be applied on a wide array of organisms.

Authors' contribution

CD and PS contributed the initial concept for this work. CD, PS, MS, MR, and LJR participated in the design of the study and performed the analysis. Paper was written with insights from the other authors. All authors read and approved the final manuscript.

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Tables

Table 1. Predicted subcellular distribution of human hypothetical proteins, from the 2006 and 2008 datasets. pTarget was used to predict the subcellular localization of 5860 and 1455 hypothetical proteins from the 2006 and 2008 datasets respectively.

Compartment	Predicted subcellular distribution 2006 dataset (5860 proteins)	Predicted subcellular distribution 2008 dataset of hypothetical proteins (1455 proteins)
Nucleus	37 %	36 %
Cytoplasm	13 %	14 %
Plasma membrane	12 %	8 %
Lysosomes	9 %	9 %
Golgi	9 %	11 %
Peroxysomes	7 %	10 %
Extracellular/Secretory	6 %	4 %
Mitochondria	5 %	5 %
Endoplasmic reticulum	2 %	3 %

Table 2. A selection of subcellular localization prediction programs for eukaryotic proteins. Listed are the numbers of compartments each program can predict targeting to, and the reported accuracy of the prediction.

Classification method	Number of localization sites	Accuracy
WoLF PSORT [14] (http://wolfpsort.org/)	12	70%
LOCtree [15] (http://cubic.bioc.columbia.edu/services/loctree/)	4	74%
BaCelLo [16] (http://gpcr.biocomp.unibo.it/bacello/)	4-5	67-76%
PA-SUB [17] (http://www.cs.ualberta.ca/~bioinfo/PA/Sub/)	11	81-94%
MultiLoc [18] (http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc/)	11	75%
pTarget [19, 20] (http://bioapps.rit.albany.edu/pTARGET/)	9	68-87%
TargetP [21] (http://www.cbs.dtu.dk/services/TargetP/)	3	90%

Table 3: Hypothetical proteins from the 2006 dataset sorted into groups depending on the probability of having a mitochondrial N-terminal presequence localization signal. Proteins of Group I, have been predicted by TargetP to belong to reliability class 1, indicating the strongest prediction. Proteins of Group II contains proteins belonging to reliability class 1 and 2, while proteins of Group III contains proteins belonging to reliability class 1, 2 and 3. All proteins of Group I, II and III have identifiable protein domains according to SMART. The three groups have been compared with all 5860 proteins of the 2006 dataset, and with their respective 2008 annotations, to evaluate whether the proteins have been characterized as being mitochondrial or have been removed.

Group	Localization signal / protein domain	Hypothetical proteins	Characterized proteins	Removed proteins	Characterized mitochondrial proteins
I	20	30% (6 of 20)	65% (13 of 20)	5% (1 of 20)	85% (11 of 13)
II	56	27% (15 of 56)	64% (36 of 56)	9% (5 of 56)	58% (21 of 36)
III	100	36% (36 of 100)	53% (53 of 100)	11% (11 of 100)	45% (24 of 53)
2006 dataset	-	25% (1455 of 5860)	21% (1215 of 5860)	54% (3190 of 5860)	6% (67 of 1214)

Table 4. Description of protein domains identified in 6 hypothetical proteins of Group I, predicted to be expressed and to have a role in a mitochondrial context. In 4 out of 6 proteins, the identified protein domains have been described in experimentally characterized proteins of the mitochondria (First 4 domains).

Accession	Domain	Description of protein domains
NP_001036096	Complex-1-LYR	This hypothetical protein contains a Complex-1-LYR domain. The domain is present in a family of proteins, which include mitochondrial proteins from NADH-ubiquinone oxidoreductase complex 1. The domain is also present in the *Saccharomyces cerevisiae* protein Isd11, which is located in the mitochondrial matrix associated with the inner membrane. Isd11 protein is a subunit of the mitochondrial Fe/S protein biogenesis [24, 25]
NP_077025	Methyltransf 12	Methyltransferase 12 domain is present in proteins, which actively transfer methyl from ubiguitous S-adenosyl-L-methionine (SAM) to nitrogen, oxygen or carbon. This methyltrasferase domain is found in a variety of SAM-dependent methyltransferases including Coq3 methyltransferase, which is a mitochondrial protein involved in ubiquinone biosynthesis. Coq3 protein is located in the matrix of the mitochondria [26-28]
NP_055588	Sel1	Sel1 like repeats are tetratricopeptide repeats (TPR) identified in LIN-12 proteins of *Caenorhabditis elegans* as a negative regulator of the Notch pathway [24] TPR-repeats are found in a variety of proteins including eukaryotic chaperone complexes involving HSP70 and HSP90, and TPRs are also present in the mitochondrial Tom70 import receptor [30, 31]
EAW75090	DUF1640	DUF1640 domain is found in proteins of unknown functions. In *Saccharomyces cerevisiae* a protein containing the domain is named FMP32 (Found in mitochondrial proteome protein 32) and was localized to the mitochondria. [uniprot.org]
NP_612455	DUF143	DUF143 : This domain has no known function and is found in the *iojap* protein of maize. The protein has no known function [32]
EAW74251	Trm112p	Trm112p is a zinc finger domain found in the TRM112 protein that is required for tRNA methylation in *Saccharomyces cerevisae*. [33]

Additional files

Additional file 1 – 2006 & 2008 Dataset

Protein entries of the 2006 and 2008 dataset. File is viewed with Excel or compatible program

Additional files provided with this submission:

Additional file 1: supplementary data.xls, 590K
http://www.biomedcentral.com/imedia/4773939826685953/supp1.xls

Paper V

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Repair of DNA damage induced by anthanthrene, a polycyclic aromatic hydrocarbon (PAH) without bay or fjord regions

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants, formed during incomplete burning of coal, oil and gas. Several PAHs have carcinogenic and mutagenic potencies, but these compounds must be activated in order to exert their mutagenic effects. One of the principal pathways proposed for metabolic activation of PAHs involves the cytochrome P450 enzymes. The DNA damaging potential of cytochrome P450-activated PAHs is generally associated with their bay and fjord regions, and the DNA repair response of PAHs containing such regions has been thoroughly studied. However, little is known about the repair of DNA damage resulting from metabolites from PAHs without bay and fjord regions. We have investigated the six-ringed PAH anthanthrene (dibenzo[*def*,*mno*]chrysene), which does not posses bay or fjord motifs. We analyzed the repair profile of human cell extracts and of cell cultures in response to DNA damage induced by cytochrome P450-activated anthanthrene. In cell extracts, functional nucleotide excision repair (NER) and mismatch repair (MMR) activities were necessary to trigger a response to anthanthrene metabolite-induced DNA damage. In cell cultures, NER was responsible for the repair of anthanthrene metabolite-induced DNA damage. However, when the NER pathway was inactivated, a residual repair pathway performed the DNA repair.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) consist of a large group of environmental pollutants, produced primarily by incomplete combustion of organic material, including burning of fossil fuels and tobacco. There are three principal pathways for metabolic activation of PAHs: the formation of PAH-*o*-quinones by dihydrodiol dehydrogenase-catalyzed oxidation, the formation of a PAH radical cation in a metabolic oxidation process involving cytochrome P450 peroxidase and finally the creation of dihydrodiol epoxides, catalyzed by cytochrome P450 enzymes. Of the three pathways, the latter is believed to be the dominant mechanism of chemical carcinogenesis of PAHs, while the formation of PAH radical cations is believed only to constitute a very small fraction of metabolites formed *in vivo* (reviewed in [1]). The formation of dihydrodiol epoxides is associated with the structure motives referred to as bay and fjord regions (Fig. 1). These motives are believed to sterically hinder epoxide hydrolase that normally removes epoxides. Bay and fjord regions, therefore, facilitate the creation of metabolites that are able react with DNA, creating bulky adducts [2]. In human cells, these types of DNA damage are presumed to be repaired by nucleotide excision repair (NER) and to a lesser extend, base excision repair (BER) [3–6]. The formation of PAH-*o*-quinones is capable of generating reactive oxidative species, which in turn are able to result in oxidative DNA damage [1].

The DNA damaging potential of metabolites from PAHs without bay and fjord regions has been reported [3], but little is known about the repair of the DNA damage resulting from metabolites of this particular group of PAHs. The six-ringed PAH anthanthrene (dibenzo[*def*,*mno*]chrysene) is a PAH without bay or fjord regions. The compound has been demonstrated to be a strong carcinogen on mouse skin [7].

According to the three principal pathways for metabolic activation of PAHs, the DNA damaging potential of a PAH without bay and fjord regions is more likely to be related to oxidative DNA damage than the creation of bulky adducts.

Research by Platt et al. has resulted in a detailed model for metabolic activation of anthanthrene and resulting derivatives with mutagenic potential [8]. Phenol derivatives were produced in the metabolization process, and anthanthrene metabolites were suggested to create stable radical species that could cause production

Abbreviations: PAHs, polycyclic aromatic hydrocarbons; NER, nucleotide excision repair; MMR, mismatch repair; BER, base excision repair; GGR, global genomic repair; TCR, transcription coupled repair; AP, apurinic; XPA, xeroderma pigmentosum complementation group A; XPC, xeroderma pigmentosum complementation group C.

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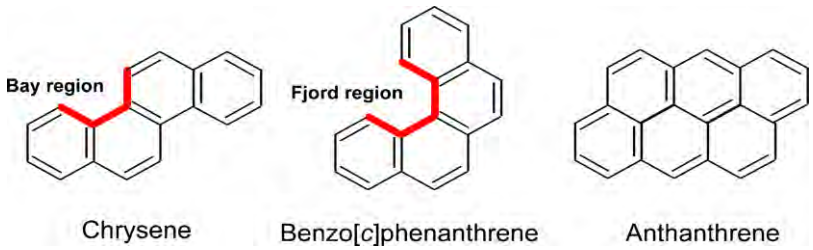

Fig. 1. Illustration of bay and fjord regions in, respectively, chrysene and benzo[*c*]phenanthrene together with anthanthrene without either regions.

of reactive species in the cell, in turn causing alkylation of DNA and oxidative DNA damage [8,9]. Recently the same group reported that the genotoxicity of anthanthrene metabolites and other PAHs were only detectable when V79 hamster cells were treated with hydroxyurea and 1-β-D-arabinofuranoside [10].

The NER pathway recognizes DNA lesions that cause structural deformation of the DNA helix. Bulky adducts are, therefore, generally considered to be repaired by NER. Following recognition, dual endonucleolytic incision around the site of the lesion is performed, 25–32 nucleotides of the damaged strand of DNA are excised and the strand is resynthesized and ligated [11–13]. The NER pathway is divided into two sub-pathways: global genomic repair (GGR), which repairs both transcribed and non-transcribed regions, and transcription coupled repair (TCR), which only acts on transcribed sequences after RNA polymerase II stalling [14]. The NER pathway is complex, requiring more than 20 different proteins, including several of the xeroderma pigmentosum (XP) complementation groups. In this article, we utilize two NER-deficient cell lines expressing defective XPC or XPA proteins (xeroderma pigmentosum groups C and A), respectively. XPC is involved in the GGR sub-pathway, where it functions in a complex that binds to the DNA lesion leading to bending of DNA, facilitating downstream NER activity. Thus, the XPC mutant is GGR-deficient but TCR-proficient. XPA is involved in verifying the initial damage recognition triggering the excision of the damaged strand of DNA. This process is identical for both GGR and TCR and the XPA mutant is, therefore, GGR- and TCR-deficient.

Due to the formation of bulky adducts, NER is considered the main repair pathway involved in repair of DNA damage caused by PAHs. However, BER has been implicated in repair of DNA damage induced by PAHs, in competition with NER [5]. The BER pathway deals with smaller damage to individual bases, such as oxidation, methylation, depurination, and deamination [15]. The BER pathway is initiated by DNA glycosylases that are capable of recognizing and removing particular subsets of base alterations by creating an apurinic (AP) site [16]. After DNA glycosylase activity, the resulting AP site must be processed by an AP endonuclease in order to generate a free 3′-OH terminus allowing insertion of a nucleotide and ligation of the strand.

With the suggestion of anthanthrene metabolites creating PAH-*o*-quinones and thereby produce reactive oxygen species [7,8], mismatch repair (MMR) must also be considered when analyzing the nature of repair of PAHs without bay and fjord regions. MMR is a post-replicative repair system, which corrects strand-specific base mispairs and small loop structures [16–19]. MMR also recognizes smaller base analogous as mispairs, which gives the repair system a role in the repair of oxidative DNA damage, thus making the system a candidate for repair of PAH-mediated DNA damage [20]. MMR is initiated by the binding of mismatch recognizing complexes to base–base mismatches and insertion/deletion loops. Following initiation, the newly synthesized strand is removed by exonuclease activity. Strand discrimination is signaled by a nick located either 3′ or 5′ to the mismatch [21] and it has been suggested that gaps between individual Okazaki fragments serve as the strand discriminating signal [22]. Finally, the strand is resynthesized and ligated.

In this work, we have used hMLH1-deficient cells to study the cellular response of MMR-deficiency on anthanthrene-induced DNA damage. The hMLH1 protein is essential for initiation of MMR, and cells deficient in hMLH1 activity are MMR-deficient.

As mentioned previously, anthanthrene is a PAH without bay or fjord regions. With the mutagenic derivatives of anthanthrene described by Platt et al. [8], we aimed to characterize the repair pathways responsible for repairing DNA damage caused by PAH. DNA damage induced by anthanthrene metabolites have recently been reported to only be detectable when dNTP synthesis was inhibited with hydroxyurea and cytosine 1-β-D-arabinofuranoside. By inhibiting the dNTP synthesis, the final step of NER, the resynthesis of the excised damaged DNA strand, is indirectly inhibited, leaving nicks in the DNA quantifiable by comet assay. However, inhibition of dNTP synthesis also inhibits the final step of MMR, which also leave nicks in the DNA. Furthermore, inhibition of dNTP synthesis can induce DNA strand breaks by stalling of replication fork, which in turn will induce other repair mechanisms [23].

We investigated DNA repair of PAH-induced damage in extracts of human cell lines with different repair status. We hypothesize that DNA damage induced by anthanthrene metabolites is repaired by both NER and an unidentified repair pathway. The NER pathway has higher affinity for PAH-induced DNA damage but when the NER pathway is inactivated, the unidentified repair pathway is able to perform a faster repair than the NER pathway.

2. Materials and methods

2.1. Cell cultures

Human fibroblast cell lines SV40-transformed "wild type" (catalogue number AG07217), SV40-transformed XPA$^{-/-}$ (catalogue number GM04429F) and SV40-transformed XPC$^{-/-}$ (catalogue number GM16093) were purchased from Coriell Cell Repositories (United States) and maintained in Dulbecco's MEM1 media supplemented with Glutamax (Invitrogen, Gibco), 10% fetal bovine serum (FBS) (Biochrom KG), and 1% penicillin/streptomycin (Invitrogen, Gibco). Human colon carcinoma cell lines HCT116 (hMLH1$^{-/-}$) and HCT116chr3 (hMLH1^{+}) [24] were maintained in McCoy's 5A media supplemented with Glutamax (Invitrogen, Gibco), 10% FBS (BioChrom AG), and 1% penicillin/streptomycin (Invitrogen, Gibco). HCT116chr3 cells were also supplemented with 400 μg/mL geneticin (G418) (Invitrogen, Gibco). Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. Throughout the remaining article, the cell lines obtained from Coriell Cell Repositories will be referred to by their catalogue numbers for simplicity, while the HCT116 strains will be referred to directly.

2.2. Cell survival following UV-radiation

Cells from exponentially growing cultures were seeded at densities of 500 and 5000 cells per 35 mm cell culture dish and allowed to

attach for 18 h. After initial incubation the media was removed and cells were exposed to UV-radiation ranging from 0 to 25 J/m^2 under a 254-nm germicidal UV lamp. After radiation growth media was added to the culture flasks and cells were grown for 9 days, with change of growth media every 2–3 days. The cell culture plates were washed with PBS (Gibco BRL, Life Technologies) and dried at room temperature for 12–24 h. Cells were stained with a mixture of 5% Giesma (Merck) and 50% methanol for 60 min and rinsed with water. Colonies were examined and counted using a stereo magnifier (Olympus SZ-ST).

2.3. Preparation of whole cell extracts for DNA repair assays

Cell-free extracts were prepared as described in [25]. In brief, cells were pelleted and resuspended in ice cold isotonic buffer (20 mM Hepes, pH 7.9, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, and 250 mM sucrose) and centrifuged at 750 × *g* for 4 min at 4 °C. Cells were washed in ice cold hypotonic buffer (20 mM Hepes, pH 7.9, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and 0.1 mM PMSF), and resuspended in ice cold hypotonic buffer to a cell density of approximately 1×10^8 cells/mL. Cells were allowed to swell for 15 min on ice and were subsequently homogenized with a minimum of four strokes of a sterile A-pestle (Kontes) followed by microscopical examination. Homogenizing was repeated until approximately 85% of the cells showed signs of membrane degradation. After homogenization, suspensions were incubated 30 min on ice. Suspensions were centrifuged at 2000 × *g* for 10 min at 4 °C, and the supernatants were centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatants were collected in a single tube and mixed carefully to ensure homogeneity. Protein concentration of extracts was determined by BioRad Protein Assay (BioRad) using BSA as standard.

2.4. Activation of anthanthrene

Anthanthrene was metabolized using a cytochrome P450 system extracted from male Sprague–Dawley rat liver as microsomes (BD Gentest). Microsomes were diluted to a 0.2-mg/mL concentration in Dulbecco's PBS solution for cell-free DNA assays or in Dulbecco's MEM1 media with Glutamax or McCoy's 5A media with Glutamax for cell culture assays. Microsomes were supplemented with a NADPH regenerating system consisting of 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 3.3 mM $MgCl_2$ and 0.4 U/mL glucose-6-phosphate dehydrogenase (BD Gentest). The metabolizing system was preincubated 5 min in a shaking water bath at 37 °C in the dark. After preincubation, anthanthrene (Fluka, 99%) in DMSO was added to a final concentration of 16 μM. The solution was incubated 100 min with shaking in a water bath at 37 °C in the dark. Mock solution was prepared as described above, but without anthanthrene.

2.5. Analysis of DNA damage and repair by human cell extracts

The pcDNA3.1 (Invitrogen) and pcDNA3.1-lacZ (Invitrogen) plasmids were purified from *Escherichia coli* DH10B using Qiagen high speed plasmid maxi-prep kit according to manufacturers instructions. The quality of the purified plasmids was controlled by visualization on a 0.7% agarose gel and plasmid concentration determined by Abs^{260} photometric scanning in a Genequant photometer (Pharmacia). 5 μg pcDNA3.1 plasmid DNA was incubated in a 100-μL solution containing TE buffer, 20% ethanol and various concentrations of native or metabolized anthanthrene, resulting in final anthanthrene concentrations of 0.3, 3.0 and 5.0 μM. All samples were incubated for 2.5 h with shaking in a thermostated water bath at 37 °C in the dark. After incubation, the DNA was ethanol precipitated according to standard methods and resuspended in TE buffer to a final concentration of 500 ng/μL DNA.

Repair reactions were prepared as described in [25]. 500 ng of pcDNA3.1-lacZ and pcDNA3.1 treated with either native anthanthrene, anthanthrene metabolites or UV light from a 254-nm germicidal UV lamp (400 J/m^2), were incubated with 140 μg protein extract in a total volume of 75 μL (30 mM Hepes (pH 7.8), 7 mM $MgCl_2$, 4 mM ATP, 200 μM CTP, GTP and UTP, 100 μM dATP, dGTP, dTTP and dCTP and 15 nM [α-^{32}P] dCTP (3000 Ci/mmol), 40 mM creatine phosphate, 100 μg/mL creatine phosphokinase, 15 mM sodium phosphate (pH 7.5)). The repair reactions were incubated 60 min at 37 °C. The reactions were terminated by adding Proteinase K (2 mg/mL), SDS (2 vol.%), and EDTA (50 mM, pH 8.0) and incubated for 30 min at 37 °C.

Plasmid DNA was extracted from the repair reaction mix using phenol extraction and ethanol precipitation according to standard methods. The plasmids were digested with BamHI and fragments visualized and quantified on a 1% agarose gel. The gel was dried in a BioRad 583 slap drier (BioRad) and placed on a PhosphorImaging screen (Amersham). The PhosphorImaging screen was analyzed using a STORM 840 scanner (Molecular Dynamics, Amersham), and the visible phosphorescence bands were analyzed with Imagemaster Total-lab software (Molecular Dynamics, Amersham). Measured amount of incorporated radioactivity was normalized to quantity of DNA.

2.6. Analysis of DNA damage and repair in cell cultures

1.5×10^5 cells were plated in 2 mL cell culture dishes (Nunc) 2 days prior to the experiment. Anthanthrene was metabolized as described earlier. Cells were incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 2.5 h in growth medium containing either 0 μM or 16.1 μM metabolized anthanthrene. Following incubation, cells were washed twice with PBS (Invitrogen, Gibco), and immediately processed for comet assay or incubated in growth media for either 30 min, 2.5 h or 14 h at 37 °C and 5% CO_2 to allow repair before being processed for comet assay.

2.7. Comet assay

A modified version [26] of single-cell gel electrophoresis was used to analyze DNA damage and repair. Twenty-five microliters of the prepared cell suspensions were mixed with 225 μL molten (42 °C) 0.75% LMP agarose (GibcoBRL, Life Technologies), and 180 μL of the resulting mixture was immediately cast on the hydrophilic side of 85 mm × 100 mm GelBond film (Medinova Scientific, Hellerup, Denmark), using chambers from Lab-Tek II Chamber Slides (Nunc, Life Technologies) as molds. The gels were cooled for approximately 10 min at 4 °C in the dark and the Lab-Tek II chambers removed. The gels were submersed in cold lysis buffer (2.5 M NaCl, 10 mM Tris-Base, 100 mM EDTA-Na_2, 1% Na-sarcosinat, 10% DMSO, 1% Triton X-100, pH 10) for 60 min, carefully washed three times with millipore water, and submersed in electrophoresis buffer (0.3 M NaOH, 1 mM EDTA-Na_2, pH 13.2) for 40 min before electrophoresis for 20 min at 300 mA and 25 V. After electrophoresis, samples were submerged twice in neutralization buffer (0.4 M Tris, pH 7.5) for 5 min and washed once with sterile water before they were fixed in 96% ethanol for approximately 90 min. The gels were air-dried overnight, dyed with SYBR Gold (Bie and Berntsen, Rødovre, Denmark), and analyzed under a fluorescence microscope (Dialux 22EB (Leica) microscope, Switzerland). From each gel, pictures were taken of randomly selected cells and were analyzed using Comet Assay III (Perceptive instruments) software to calculate comet tail moment. Mean tail moment was calculated from the comet tail moment of 50 randomly selected cells and each data

Table 1
Description of cell lines used. (+) Annotates proficiency and (−) deficiency.

Cell line	GGR status	TCR status	MMR status
AG07217	+	+	+
GM04429F	−	−	+
GM16093	−	+	+
HCT116chr3	+	+	+
HCT116	+	+	−

point consists of a triplicate of mean tail moments. This value was used as a quantitative index of single and double stranded DNA breaks. The statistical analysis was performed using Student's *t*-test. The differences between data sets were considered significant at *p*-values <0.05.

3. Results

We used five different human cell lines to characterize DNA repair activity caused by anthanthrene metabolites (Table 1): AG07217 (NER- and MMR-proficient), GM04429F (XPA- and NER-deficient), GM16093 (XPC- and GGR-deficient), HCT116 (MMR-deficient), and HCT116chr3 (NER- and MMR-proficient). The repair defects of the cell lines were confirmed by sensitivity to UV-radiation (NER) and resistance to N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) (MMR) [27] (data not shown).

3.1. Post-labeling assay of plasmids exposed to anthanthrene metabolites

In order to characterize DNA repair activities associated with DNA damage induced by anthanthrene metabolites, we measured the incorporation of [α-^{32}P] dCTP into a well-defined DNA substrate in a post-labeling assay. Damaged plasmid DNA was incubated with [α-^{32}P] dCTP and cell extracts from repair-deficient as well as proficient cell lines. The amount of incorporated [α-^{32}P] dCTP is an indicator of repair activity in the cell extracts, in the form of excision and resynthesis of DNA (Fig. 2). Our results suggest that plasmid DNA incubated with anthanthrene metabolites demonstrates increased incorporation of [α-^{32}P] dCTP into DNA when incubated with AG07217 NER-proficient cell extracts. The incorporation of [α-^{32}P] dCTP is proportional to the concentration of anthanthrene metabolites, and in AG07217 NER-proficient cell extracts, the activity of repair is, therefore, correlated to the concentration of anthanthrene metabolites (Fig. 2). Incubating plasmid DNA with native anthanthrene, in AG07217 NER-proficient cell extracts, did not induce a significant incorporation of the radioactive nucleotide. This indicates that activation of anthanthrene is necessary to induce DNA damage in this experimental system.

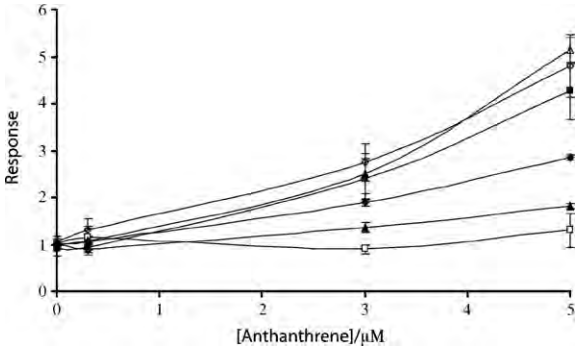

Fig. 2. Dose–response curves plotting normalized level of repair as a function of anthanthrene concentration of AG07217 extracts acting on DNA incubated with anthanthrene metabolites (■) or native anthanthrene (□), GM04429F (▲), GM16093 (△), HCT116 (♦), and HCT116chr3 (◊) extracts acting on DNA incubated with anthanthrene metabolites ($n = 3$). The value of each point in the response curves shows the ratio between the amount of linear plasmid DNA isolated from each repair action (from ethidium bromide staining of the agarose gel) and incorporated labeled dCTP (from phosphoimaging), and subsequently normalizing these values with respect to incorporation of dCTP in repair reactions with anthanthrene concentration = 0 μM (arbitrarily set to 1).

The cell extract of the GM04429F XPA-deficient cell line, showed decreased incorporation of [α-^{32}P] dCTP compared to AG07217 NER-proficient cell extracts, indicating reduced repair activity, as expected from the NER-deficient status of this cell line. We did measure some [α-^{32}P] dCTP incorporation at 5 μM anthanthrene metabolites, suggesting that either a residual NER repair activity is present, or that other repair mechanisms are initiating repair of anthanthrene-induced DNA damage when NER is inhibited. Interestingly, repair activity of the cell extract of the GGR-defective cell line GM16093 that does not express a functional XPC protein, is not significantly different from the response curve of AG07217 cell extract.

Fig. 2 shows that the repair response measured in MMR-deficient HCT116 cell extracts is lower than in the MMR-proficient HCT116chr3 cell extracts. The repair activity in HCT116chr3 cell extracts is comparable to the repair response of AG07217 wildtype extract suggesting that MMR is involved in repair of DNA damage caused by anthanthrene.

3.2. Comet assays of cell cultures exposed to anthanthrene metabolites

In order to investigate the effect of metabolized anthanthrene in cell cultures, we used the comet assay to determine the occurrence of DNA breaks after treatment with metabolized anthanthrene. We assayed the NER-proficient cell line AG07217 and the XPA- and XPC-deficient cell lines GM04429F and GM16093 immediately after treatment with metabolized anthanthrene as well as 30 min, 2.5 and 14 h after treatment to allow DNA repair. The MMR-proficient and MMR-deficient cell lines HCT116chr3 and HCT116 where only investigated immediately after treatment with metabolized anthanthrene and 2.5 h after treatment (Fig. 3). Our results showed that regardless of repair status metabolized anthanthrene-induced DNA damage in form of DNA breaks when compared to mock treated cells. The increase of DNA damage immediate after anthanthrene treatment is most prominent in HCT116chr3, AG07217 NER-proficient and GM16093 XPC-deficient cell lines with an 8–12-fold increase in DNA breaks. After treatment with metabolized anthanthrene, the NER-proficient cell line AG07217 and the XPA- and XPC-deficient cell lines GM04429F and GM16093 were allowed to repair for 14 h. After 30 min of repair, a significant 2.5-fold decrease in levels of DNA damage was found for the XPC-deficient cell line GM16093 ($p < 0.05$). No significant decrease was found for the NER-proficient cell line AG07217 or the XPA-deficient cell line GM04429F. After 2.5 h of repair, the two NER mutants GM04429F and GM16093 displayed, respectively, a 1.8- and 1.7-fold further decrease of the levels of DNA damage in form of DNA breaks ($p < 0.05$ for GM04429F and $p < 0.01$ for GM16093). For the NER-proficient cell line AG07217, no significant difference was found between the levels of DNA damage in cells immediately after treatment with metabolized anthanthrene and after 30 min and 2.5 h of repair. After 14 h of repair, the level of DNA damage was reduced to the level of mock treated cells for all investigated cell lines. This indicates that the NER functional cell lines are unable to perform repair as fast as the NER mutants.

For the MMR-deficient (HCT116) and the corresponding MMR-proficient (HCT116chr3) cell lines 2.5 h of repair did not alter the occurrence of DNA breaks significantly indicating that the cell lines

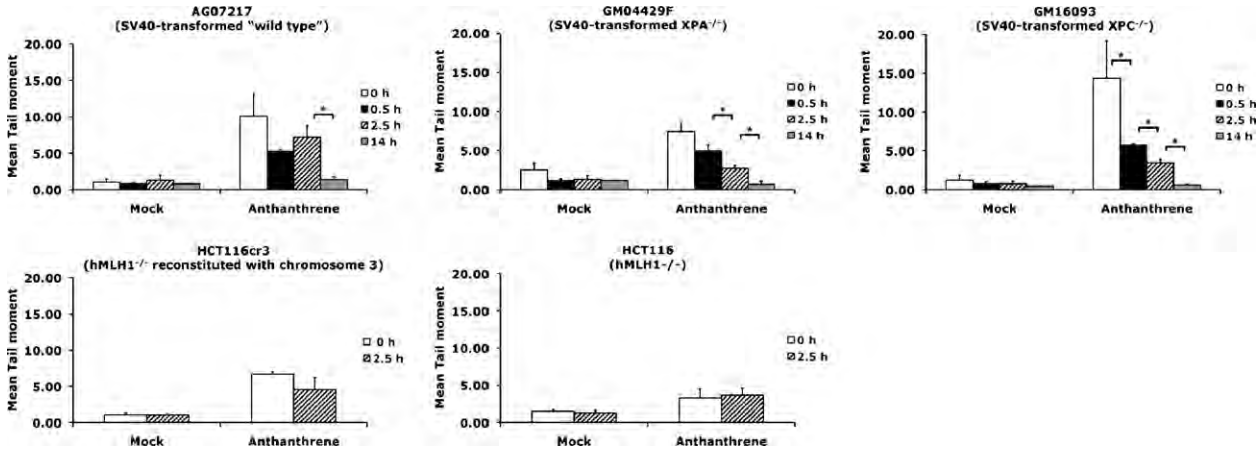

Fig. 3. Comet assay of AG07217 (NER- and MMR-proficient), GM04429F ($XPC^{-/-}$, NER-deficient), GM16093 ($XPC^{-/-}$, GGR-deficient), HCT116chr3 (NER- and MMR-proficient) and HCT116 (hMLH1, NER-proficient and MMR-deficient) cell lines, 0 h (white bar), 30 min (black bar), 2.5 h (striped bar) and 14 h (grey bar) after treatment. Cells were either treated with anthanthrene in metabolizing system (treated) or metabolizing system alone as a control (mock) (see Section 2). DNA damage was measured as mean tail moment ($n = 3$). (*) Denotes significant difference. The statistical analysis was performed using Student's *t*-test. A difference at $p < 0.05$ was considered significant.

are incapable or inefficient in repair of the DNA damage caused by anthanthrene metabolites within this timeframe.

4. Discussion

We have examined the nature of DNA repair pathways activated by anthanthrene metabolites in human cell extracts and in cell cultures using cell lines with different repair status. Our results confirm that anthanthrene has a DNA damaging effect on human cells, despite its lack of bay and fjord regions. We also show that, anthanthrene metabolites induce DNA damage that activates the NER pathway in both cell cultures and cell extracts, and implicate the MMR pathway in cell extracts. In post-labeling assays, cell extract of the NER-deficient GM04429 XPA-deficient mutant displayed decreased DNA repair, measured by incorporation of [α-^{32}P] dCTP, when compared to the NER repair-proficient AG07217 cell line. The XPA protein identifies NER lesions and recognizes various NER-specific types of damage. The protein is important in positioning the repair machinery correctly around the DNA lesion [28]. The XPA protein is, therefore, essential for the initiation of both the GGR and TCR sub-pathways of NER. Our results using cell extracts clearly indicate the involvement of the NER pathway in repairing DNA damage caused by anthanthrene metabolites. This is in accordance with results from other groups [9]. Since DNA helix distortions are the main substrates for the NER, our results indicate that anthanthrene metabolites can result in bulky adducts despite the lack of bay and fjord regions. In post-labeling assays, no difference in DNA repair was measured between XPC-deficient GM16093 and the NER-proficient AG07217 cell lines. The XPC protein is not essential for TCR and is only involved in GGR [29]. This indicates that in cell extracts, this particular pathway of NER is not involved in repair of DNA damage caused by metabolized anthanthrene.

Using the comet assay, we show that the NER-proficient cell lines AG07217, HCT116 and HCT116chr3 are unable to reduce the occurrence of DNA breaks over a period of 2.5 h, while the NER-deficient cell lines GM04429 and GM16093 were able to decrease the occurrence of DNA damage by, respectively, 2.8- and 4.2-fold in the same period of time. Furthermore, the XPC-deficient cell line GM16093 was able to decrease the levels of DNA damage in the form of DNA breaks already after 30 min of repair. This indicates that a functional NER delays the repair of DNA damage resulting from anthanthrene metabolites in cell cultures. It has been reported how two different repair systems can compete for the same substrate with the result of the slower repair pathway predominating [30]. We hypothesize that DNA damage induced by anthanthrene metabolites are subject for repair by both NER and an unidentified repair pathway, where NER has a higher affinity for the damage. When the NER pathway is impaired, the unidentified repair pathway is able to perform a faster repair than the NER pathway.

Of the investigated cell lines, the XPC-deficient cell line had the highest occurrence of DNA breaks measured by comet assay immediately after treatment with anthanthrene metabolites. Compared to mock treated cells, the XPC-deficient GM16093 cell line had a 12-fold higher occurrence of DNA breaks following anthanthrene metabolite treatment. The higher occurrence of DNA breaks can be cell specific; however, the XPC protein was recently shown to be implicated in the BER pathway in response to oxidative damage [31]. It was found that XPC stimulates the activation of the specific DNA glycosylase OGG1 in response to oxidative damage. Glycosylases recognize and incise individual damaged bases leaving abasic sites transformed to a ligatable nick by AP endonucleases. With the XPC-deficient GM16093 cell line, the stimulation of OGG1 would be absent, leading to a potentially slower activation of the BER pathway.

Although the primary function of MMR is to repair DNA replication errors its involvement in cellular responses to a variety of chemotherapeutic drugs has been demonstrated. [32]. As found by Platt and co-workers [7], a portion of the anthanthrene metabolites has oxidative properties. Even though Platt et al. did not propose that this sub-group of anthanthrene metabolites was responsible for the main mutagenic effect, it is possible that these oxidation products can cause DNA damage in the form of lesser DNA-base analogues, which is recognizable by MMR proteins as mismatches. We show that the absence of functional hMLH1 decreases the repair response in cell extracts. The same trend was not evident with cultured cells treated with metabolized anthanthrene. This indicates that the mutagenic effect of activated anthanthrene is more likely to be related to the formation of bulky adducts than the formation of oxidative DNA damage, despite the lack of bay and fjord regions.

Conflict of interest

The authors declare that there are no conflicts of interests.

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